EXHIBIT A

PERSPECTIVE SERIES

Quorum sensing | E. Peter Greenberg, Series Editor

Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections

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Traditional treatment of infectious diseases is based on compounds that aim to kill or inhibit bacterial growth. A major concern with this approach is the frequently observed development of resistance to antimicrobial compounds. The discovery of bacterial-communication systems (quorum-sensing systems), which orchestrate important temporal events during the infection process, has afforded a novel opportunity to ameliorate bacterial infection by means other than growth inhibition. Compounds able to override bacterial signaling are present in nature. Herein we discuss the known signaling mechanisms and potential antipathogenic drugs that specifically target quorum-sensing systems in a manner unlikely to pose a selective pressure for the development of resistant mutants.

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One of the greatest accomplishments of modern medicine has been the development of antimicrobial pharmaceuticals for the treatment of infectious diseases. Alexander Fleming discovered the first antibiotic, penicillin, in 1928, and after over half a century of intense research, most acute bacterial infections can be treated effectively with antibiotics. Conventional antibiotics possess broad-range efficacy via toxic or growth-inhibitory effects on target organisms. However, an increased frequency of bacterial mutations has resulted in a significantly increased incidence of antibiotic resistance. The horizontal spread of resistance genes to other bacteria of the same or different species has been shown to rapidly create bacterial populations with (a) an increased ability to degrade antibacterial compounds; (b) decreased permeability; (c) decreased affinity for the antibiotic; or, finally, (d) increased efflux of many different antibiotics (1, 2). The increasing occurrence of multiresistant pathogenic bacterial strains has gradually rendered traditional antimicrobial treatment ineffective. Today, a global concern has emerged that we are entering a post-antibiotic era with a reduced capability to combat microbes, and, hence, the development of novel therapeutic approaches to the treatment of bacterial infections constitutes a focal point of modern research. The alternative to antibiotic-mediated bacteria killing or growth inhibition is attenuation of bacterial virulence such that the organism fails to establish successful infection and, in consequence, is cleared by the host immune response. Compounds with such abilities are the result of rational drug design and are termed antipathogenic drugs as opposed to antibacterial drugs (i.e., most traditional antibiotics). Antipathogenic drugs target key regulatory bacterial systems that govern the expression of virulence factors.

In recent years, researchers have come to appreciate that, in nature, most bacteria form complex surface-attached (sessile) communities called biofilms. Bacteria present within biofilms have characteristics distinct from those of free-swimming (planktonic) bacteria of the same species, including a significantly increased tolerance to antimicrobial therapies and the host immune response (3). In modern clinical microbiology, the establishment of bacterial biofilms is often considered a pathogenicity trait during chronic infections (4). Biofilm formation is an example of microbial community behavior. Both Gram-positive and Gram-negative bacteria have been found to coordinate this behavior through cell-to-cell communication mediated by small, diffusible signals. This phenomenon has been termed quorum sensing and is prevalent among both symbiotic and pathogenic bacteria associated with plants and animals. Many of the phenotypes regulated by cell-to-cell communication are involved in bacterial colonization and virulence.

Among the Gram-negative bacteria, the most well studied quorum-sensing system is the LuxR-LuxI homologous system and the cognate signal molecules: *N*-acyl-homoserine lactones (AHLs) (3). This quorum-sensing system is widespread among Gram-negative genera and is involved in the regulation of many host-associated phenotypes, including production of viru-

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Conflict of interest: Michael Givskov is the founder and vice president of QSI Pharma A/S (Lyngby, Denmark).

Nonstandard abbreviations used: *N*-acyl-homoserine lactone (AHL); exopolymeric substance (EPS); autoinducer 2 (AI-2); *S*-adenosyl methionine (SAM); *Pseudomonas* quinolone signal (PQS).

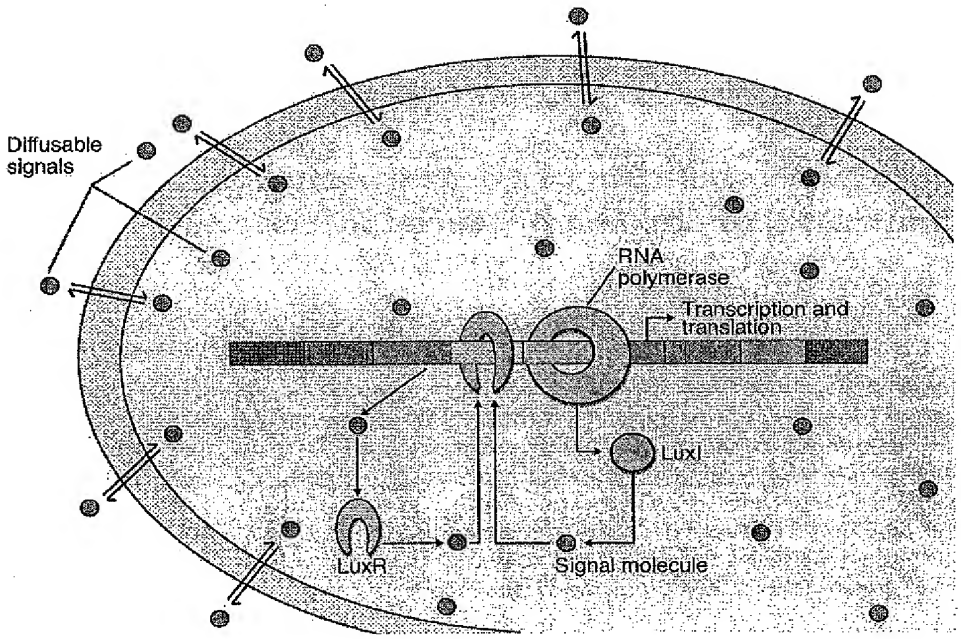

Figure 1
The archetypical Lux quorum sensor. The AHL signal (green circles) is synthesized by the *luxI* gene product LuxI (the synthase). At a certain threshold concentration, the AHL signal interacts with the receptor LuxR (encoded by *luxR*), which binds to the promoter sequence of the target genes (in this case, the *lux* operon) and in conjunction with the RNA polymerase promotes transcription.

lence factors (5–7) and secondary metabolites (8). Emerging evidence points to the involvement of quorum sensing in biofilm formation and surface motility in the opportunistic pathogens *Pseudomonas aeruginosa* (9), *Burkholderia cepacia* (10), and *Aeromonas hydrophila* (11). These observations suggest that quorum sensing serves to link biofilm formation with virulence factor production. Interestingly, AHL-based cross-talk has been demonstrated between *P. aeruginosa* and *B. cepacia* (12) and between *S. liquefaciens* and *P. aeruginosa* (13).

The observation that quorum sensing is linked to virulence factor production and biofilm formation suggests that many virulent Gram-negative organisms could potentially be rendered nonpathogenic by inhibition of their quorum-sensing systems. Research into quorum sensing, and inhibition thereof, may provide a means of treating many common and damaging chronic infections without the use of growth-inhibitory agents, such as antibiotics, preservatives, and disinfectants, that unavoidably select for resistant organisms.

AHL-mediated quorum sensing

Quorum sensing is a generic regulatory mechanism used by many Gram-negative bacteria and Gram-positive bacteria to perceive and respond to factors as varied as changing microbial population density and the expression of specific genes. The concentration of a signal molecule reflects the density of bacterial cells in a defined environment, and the perception of a threshold level of that signal indicates that the population is "quorate," i.e., sufficiently dense to make a behavioral group–based decision. Quorum sensing is thought to afford pathogenic bacteria a mechanism to minimize host immune responses by delaying the production of tissue-damaging virulence factors until sufficient bacteria have amassed and are prepared to overwhelm host defense mechanisms and establish infection. In our laboratory, we also view quorum sensing as a mechanism by which bacteria expose part of their genetic repertoire for recognition by other organisms (prokaryotes as well as eukaryotes): a phenomenon referred to as cross-talk. One environment that contains a large number of bacteria in close proximity is the bacterial biofilm. Furthermore, the dense and diffusion-limited biofilm matrix seems to provide ideal conditions for accumulation of signal molecules and a protected environment for bacteria to induce quorum sensing–regulated virulence factors and launch an attack on the host.

AHL-mediated quorum-sensing systems are found in a large number of Gram-negative bacterial species belonging to the α, β, and γ subclasses of proteobacteria, including bacteria in the genera *Agrobacterium*, *Aeromonas*, *Burkholderia*, *Chromobacterium*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Hafnia*, *Nitrosomonas*, *Obesumbacterium*, *Pantoea*, *Pseudomonas*, *Rahnella*, *Ralstonia*, *Rhodobacter*, *Rhizobium*, *Serratia*, *Vibrio*, *Xenorhabdus*, and *Yersinia* (reviewed in ref. 3). The quorum-sensing system consists, in brief, of a four-component circuit: an AHL signal molecule, a LuxI-type signal synthase, a LuxR-type signal receptor, and the target gene(s) (Figure 1). The AHL signal is synthesized at a low basal level by the AHL synthase. AHL signals diffuse out of the bacteria and into the surrounding environment. An increase in

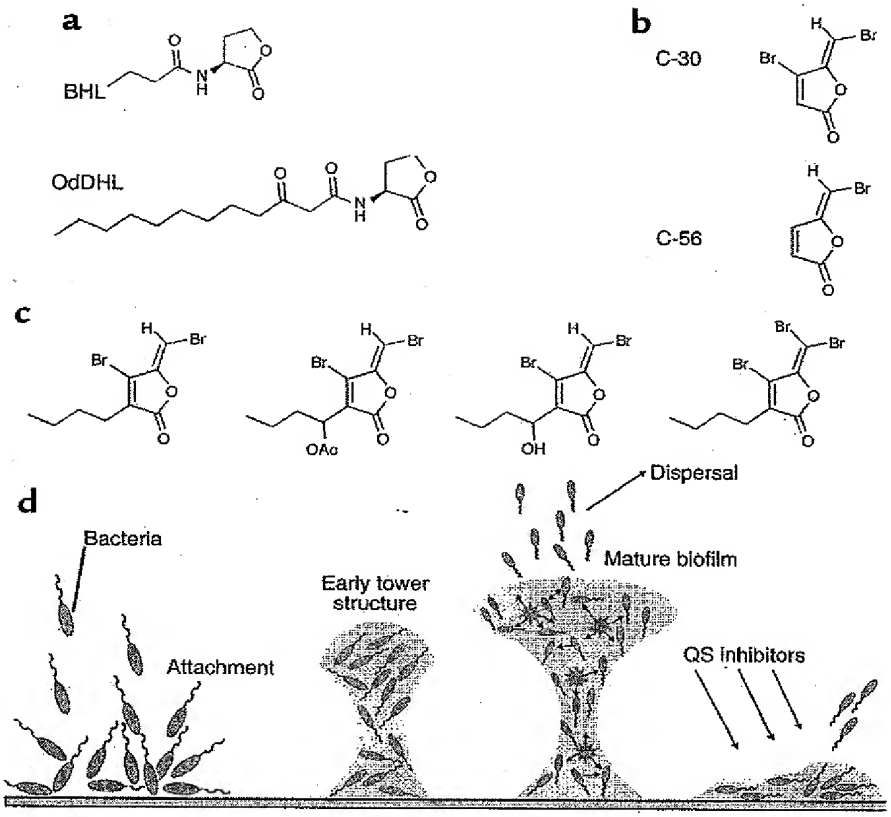

Figure 2
(**a**) Molecular structures of the two cognate signal molecules produced by *P. aeruginosa*, BHL ([*N*-butyryl]-L-homoserine lactone), and OdDHL (*N*-[3-oxo-dodecanoyl]-L-homoserine lactone). (**b**) Synthetic quorum-sensing (QS) inhibitors derived from (**c**) natural brominated furanone compounds isolated from *D. pulchra*. (**d**) Temporal biofilm development and dispersal. Stars represent QS.

bacterial population density leads to an increase in local AHL concentration, and, at a threshold concentration, this signal interacts with a cognate receptor (LuxR-type response regulator) that in turn is activated as a positive transcription factor and modulates the expression of quorum sensing–regulated genes. Often, the quorum sensor is subject to autoinduction, because the gene encoding the signal synthase is among the target genes; hence, a positive feedback regulatory loop is created. The autoinduction allows a rapid increase in signal production and dissemination, which in turn induces a quorum sensing–controlled phenotype throughout the bacterial population (see Figure 1).

Microbial biofilms and chronic infections

Biofilms are now considered ubiquitous in the natural world (14). Bacterial biofilms have been observed to be extremely heterogeneous, both structurally and with regard to the physiology of the bacterial cells within them. The prevailing conceptual model depicts bacterial biofilms as being made up of microcolonies, which serve as the basic unit of the greater biofilm structure. Microcolonies are hydrated structures consisting of bacterial cells enmeshed in a matrix of exopolymeric substances (EPSs). Bacteria may proliferate on the attachment surface, leading to microcolony expansion. Eventually, community growth becomes limited by substrate availability due to increased diffusion distances, and the biofilm reaches a steady state. Such mature biofilms often consist of "towers" and "mushrooms" of cells in an EPS matrix (Figure 2). Interstitial voids and channels separate the biofilm structures and facilitate a convective flow in order to transport nutrients to interior parts of the biofilm and remove waste products. The biofilm mode of growth has been shown to facilitate bacterial survival in a variety of environmental stresses, including antibiotics and disinfectants (4, 15). Biofilms have become evident in many, if not most, environmental, industrial, and medical bacteria-related problems. A recent public announcement from the NIH stated that more than 60% of all microbial infections involve biofilms (1).

Quorum sensing as a target for antimicrobial therapy

Given the many bacteria that employ quorum sensing in the control of virulence, quorum sensing constitutes a novel target for directed drug design (16). While AHL-mediated quorum-sensing systems are employed by Gram-negative bacteria, many Gram-positive bacteria,

including *Bacillus subtilis*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*, use small peptides or modified peptides for signaling. Autoinducer 2 (AI-2), a signaling molecule common to many diverse bacteria, has recently been described (17). Genome sequencing revealed the presence of *luxS* homologs (encoding the AI-2 signal synthase) in many pathogens, including *Escherichia coli*, *Helicobacter*, *Neisseria*, *Porphyromonas*, *Proteus*, *Salmonella*, *Enterococcus faecalis*, *Streptococcus pyogenes*, and *S. aureus* (18). In some bacteria, AI-2 signaling is required for virulence (19), but in other bacteria, it does not appear essential for bacterial virulence (20). Recently, Winzer and colleagues suggested that, in most bacteria, AI-2 is simply a metabolic by-product and, therefore, doubtful as a drug target (21).

Quorum sensing–inhibitory compounds might constitute a new generation of antimicrobial agents with applications in many fields, including medicine (human and veterinary), agriculture, and aquaculture, and the associated commercial interests are substantial. Indeed, in recent years a number of biotechnology companies that aim specifically at developing anti–quorum-sensing and anti-biofilm drugs have emerged (QSI Pharma A/S, Lyngby, Denmark; Microbia, Cambridge, Massachusetts, USA; Quorex Pharmaceuticals Inc., Carlsbad, California, USA; and 4SC AG, Martinsried, Germany). Several strategies aiming at the interruption of bacterial quorum-sensing circuits are possible, including (a) inhibition of AHL signal generation, (b) inhibition of AHL signal dissemination, and (c) inhibition of AHL signal reception.

Inhibition of AHL signal generation

The vast majority of bacteria that produce AHL signals encode one or more genes homologous to *luxI* of *Vibrio fischeri* (see Figure 1). Expression of these genes in heterologous host backgrounds has demonstrated that the LuxI-type protein is required and sufficient for production of AHL signals. The catalysis of AHL synthesis has been studied in vitro for three LuxI family members. The reaction involves a sequentially ordered reaction mechanism that uses *S*-adenosyl methionine (SAM) as the amino donor for generation of the homoserine lactone ring moiety, and an appropriately charged acyl carrier protein (ACP) as the precursor for the acyl side chain of the AHL signal (22).

Knowledge about signal generation can be exploited to develop quorum-sensing inhibitor molecules that target AHL signal generation. Various analogs of SAM, such as *S*-adenosylhomocysteine, *S*-adenosylcysteine, and sinefungin, have been demonstrated to be potent inhibitors of AHL synthesis catalyzed by the *P. aeruginosa* RhlI protein (22). The reaction chemistry of AHL synthase with SAM appears to be unique, even though SAM is a necessary and common intermediate in many prokaryotic and eukaryotic pathways. This raises the hope that SAM analogs could be used as specific inhibitors of quorum-sensing signal generation, without affecting eukaryotic enzymes that use SAM as a substrate. Some recent reports have demonstrated that certain macrolide antibiotics are capable of repressing *P. aeruginosa* AHL synthesis when applied at subminimal growth-inhibitory concentrations (23, 24). Erythromycin has been reported to suppress production of *P. aeruginosa* hemagglutinins, protease, hemolysin, and AHL signals (25). Macrolide antibiotics are generally recognized as inhibitors of protein synthesis at the ribosomal level. It remains unclear how these antibiotics interfere with quorum-sensing circuits. It is also unclear how resistance to these antibiotics affects their quorum sensing–modulatory properties.

Inhibition of AHL signal dissemination

Bacterial cell-to-cell communication can be inhibited by a decrease in the active signal-molecule concentration in the environment. AHL decay might be a consequence of a nonenzymatic reaction; e.g., AHL signals are subject to alkaline hydrolysis at high pH values (26). Some bacteria have been reported to specifically degrade AHL signals (27, 28). Dong et al. (27) found a *Bacillus* species that produced an enzyme, termed AiiA, that catalyzed the hydrolysis of AHL molecules. Expression of the *aiiA* gene in the plant pathogen *Erwinia carotovora* resulted in reduced release of AHL signals, decreased extracellular pectolytic enzyme activity, and attenuated soft rot disease symptoms in all plants tested (27). Moreover, transgenic plants expressing AiiA have been shown to be significantly less susceptible to infection by *E. carotovora* (29). In another study, a *Variovorax paradoxus* strain able to grow using 3-oxo-C6-*N*-homoserine lactone as the sole energy and nitrogen source was isolated from a soil sample (28). In our own laboratory, we have conducted similar screenings and found that bacteria able to degrade or metabolize AHL molecules can be isolated from environmental samples at a high frequency (M. Givskov et al., unpublished observations). The ecological significance of such AHL-

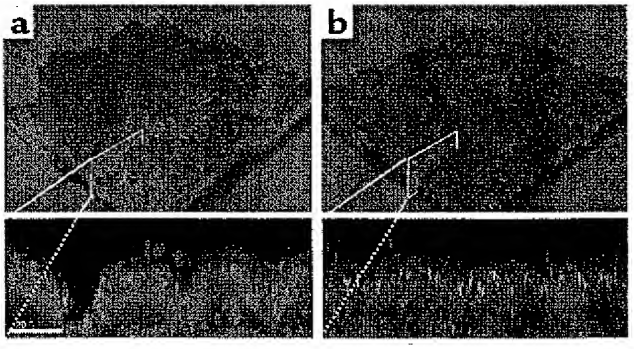

Figure 3
Furanone-treated *P. aeruginosa* biofilms are less tolerant to tobramycin. Scanning confocal laser photomicrographs of *P. aeruginosa* PAO1 biofilms grown in the absence (**a**) or the presence (**b**) of 10 μM C-30. After 3 days, the biofilms were exposed to 100 μg/ml tobramycin for 24 hours. Bacterial viability was assayed by staining using the LIVE/DEAD *Bac*Light Bacterial Viability Kit (Molecular Probes Inc., Eugene, Oregon, USA). Red areas are dead bacteria; green areas are live bacteria.

degrading bacteria is not clear, but AHL-degrading enzymes are of great clinical interest for use in the prevention of diseases caused by quorum sensing–proficient bacterial populations.

Inhibition of AHL signal reception

Blocking of quorum-sensing signal transduction can be achieved by an antagonist molecule capable of competing or interfering with the native AHL signal for binding to the LuxR-type receptor. Competitive inhibitors would conceivably be structurally similar to the native AHL signal, in order to bind to and occupy the AHL-binding site but fail to activate the LuxR-type receptor. Noncompetitive inhibitors may show little or no structural similarity to AHL signals, as these molecules bind to different sites on the receptor protein.

Several reports describe the in vitro application of AHL analogs to achieve inhibition of the quorum-sensing circuits of various bacteria (30–34). These studies have generated substantial knowledge about the structure-function relationships of AHL signals, which is of great value for the continued search for potent quorum-sensing inhibitors. The acyl side chain has been modified in several ways, and it has been shown that the length is crucial to activity (35, 36). In one study of quorum sensing in *E. carotovora*, it was reported that increasing the length of the acyl side chain by one methylene unit reduced activity by 50%, whereas a two-unit extension reduced activity by 90%. Decreasing the chain length by one methylene unit decreased activity to 10% (35). Interestingly, AHL analogs with a longer side chain than the native AHL generally appear to be more efficient inhibitors than AHL analogs with a shorter side chain. This observation might suggest that a minimum acyl side chain length determined by the native AHL signal is required for binding to LuxR homologs and that longer acyl chains can be accommodated in the AHL-binding site of LuxR-type receptors. The flexibility of the acyl side chain also appears to be important for binding to LuxR-type proteins. For instance, reduction of the chain rotation by introduction of an unsaturated bond close to the amide linkage almost completely abolishes binding to the receptor (31, 34, 35). In accordance with this suggestion, no natural AHL signal has ever been reported to contain a 2,3 unsaturated bond. A study investigating the *P. aeruginosa* LasR receptor suggested that the fully extended chain geometry is necessary for activation, whereas constrained analogs locked into different conformations showed no activity (37). The substitution at the β-position is important for the agonistic activity of AHLs, but there is no clear rule regarding the importance of this substitution to the maintenance of antagonistic activity.

The homoserine lactone moiety is generally very sensitive to modifications, and the chirality is crucial to biological activity. Natural AHL signals are L-isomers, whereas D-isomers are generally devoid of biological activity (35). The acyl side chain appears essential for activity, as exemplified in *E. carotovora*, in which the unsubstituted homoserine lactone ring fails to activate the quorum-sensing system (35). Conversion of the homoserine lactone ring to a homoserine lactame ring results in a molecule without agonistic or antagonistic properties (30, 35). Interestingly, a change of the homoserine lactone structure to a homoserine thiolactone ring appears permissible in several quorum-sensing systems (30, 31, 35). A recent study showed that LasR and RhlR proteins responded differently to changes in the homoserine lactone moiety (38). This may indicate that the two *P. aeruginosa* AHL receptors differ significantly in their AHL-binding sites.

Quorum-sensing inhibitors expressed by higher organisms

A number of reports describe the ability of higher organisms to interfere with AHL-mediated quorum sensing. The best-characterized example is that of the Australian macroalga *Delisea pulchra*, described below. Recently, another example of eukaryotic interference with AHL-mediated signaling was provided by Teplitski et al. (39), who showed that several plants secrete substances that mimic bacterial AHL signal activities and affect quorum sensing–regulated behaviors in associated bacteria. Exudates from pea (*Pisum sativum*) were demonstrated to contain several separable activities that either stimulated or inhibited bacterial AHL-dependent phenotypes (39). Many plants and fungi have coevolved and established carefully regulated symbiotic associations with bacteria. Interestingly, many plant-associated proteobacteria possess AHL-mediated quorum-sensing systems (40). Importantly, both plants and fungi are devoid of the active immune systems that are observed in mammals; rather, they rely on chemical defense systems to deal with bacteria in the environment. For these reasons, it might be expected that plants and fungi have evolved to produce chemical compounds to inhibit (or in other cases to stimulate) bacterial AHL-mediated communication. We have conducted screenings of plants (including some used in traditional herbal medicine) and fungal extracts for AHL-inhibitory activity (M. Givskov et al., unpublished results). A surprisingly large number of extracts contained quorum sensing–inhibitory activities. Not surprisingly, we found AHL-producing bacteria (which secrete hydrolytic exoenzymes) associated with these plants and their roots. We believe that the interplay of signals and signal inhibitors enables a stable coexistence of the eukaryotic host and the bacteria as long as the plant or root produces sufficient inhibitor to block the quorum-sensing systems of the colonizing organisms. Currently, work is in progress to characterize and isolate the pure compounds responsible for this quorum sensing–inhibitory activity.

Inhibition of quorum sensing by halogenated furanone compounds

The ability of bacteria to form biofilms is a major challenge for living organisms at risk of infection, such as humans, animals, and marine eukaryotes (41, 42). Marine plants are, in the absence of an advanced

immune system, prone to disease (43, 44). Bacteria can be highly detrimental to marine algae and other eukaryotes (42). The Australian red macroalga *D. pulchra* produces a range of halogenated furanone compounds (45) that display antifouling and antimicrobial properties (46–48). This particular alga originally attracted the attention of marine biologists because it was devoid of surface colonization, i.e., biofouling, unlike other plants in the same environment. Biofouling is primarily caused by marine invertebrates and plants, but bacterial biofilms are believed to be the first colonizers of submerged surfaces, providing an initial conditioning biofilm to which other marine organisms may attach (49). Therefore, the abundance and composition of the bacterial community on the surface will significantly affect the subsequent development of a macrofouling community (50, 51). Consequently, eukaryotes have developed chemical defense mechanisms (47, 52, 53) that, in several cases, include secondary metabolites that inhibit phenotypes relevant to bacterial colonization (54–56). Such secondary metabolites — furanones — are produced by the marine alga *D. pulchra* (54, 57, 58).

D. pulchra furanone compounds consist in general of a furan ring structure with a substituted acyl chain at the C-3 position and a bromine substitution at the C-4 position (see Figure 2). The substitution at the C-5 position may vary in terms of side chain structure. The natural furanone is halogenated at various positions by bromine, iodide, or chloride (45). *D. pulchra* produces at least 30 different species of halogenated furanone compounds, which are stored in specialized vesicles and are released at the surface of the thallus at a concentration ranging from 1 to 100 ng/cm^2. Field experiments have demonstrated that the surface concentration of furanones is inversely correlated to the degree of colonization by marine bacteria (55).

Givskov et al. (58) hypothesized that furanones of *D. pulchra* constitute a specific means of eukaryotic interference with bacterial signaling processes. An important discovery was the furanone-mediated displacement of radiolabeled AHL molecules from LuxR (59). This suggests that furanone compounds compete with the cognate AHL signal for the LuxR receptor site. Extensive experimental evidence in support of this model has accumulated during recent years. This includes the observations that furanones (a) repress AHL-dependent expression of *V. fischeri* bioluminescence (59); (b) inhibit AHL-controlled virulence factor production and pathogenesis in *P. aeruginosa* (60, 61); (c) inhibit quorum sensing–controlled luminescence and virulence of the black tiger prawn pathogen *Vibrio harveyi* (62); and, finally, (d) inhibit quorum sensing–controlled virulence of *E. carotovora* (63).

The natural furanone compounds have little or no effect on the quorum-sensing systems of *P. aeruginosa*. In collaboration with Staffan Kjelleberg's research group, we embarked on the process of drug development to find more potent quorum-sensing inhibitors. The natural furanone compounds were modified by chemical synthesis and screened for increased efficacy. Some derivatives of the *D. pulchra* furanone compounds were shown to repress quorum sensing in *P. aeruginosa* and reduce virulence factor expression (60, 61). Because synthetic compounds, which function well against planktonic cells, might be less efficient against biofilm bacteria, the efficacy of these quorum-sensing inhibitor compounds against bacterial biofilms was assayed. By means of AHL monitors built on the *P. aeruginosa* quorum sensors and the *lasB-gfp* target gene, the efficacy of these compounds was measured via GFP expression. The use of the GFP-based single-cell technology in combination with scanning confocal laser microscopy allowed estimation of furanone penetration and half-life and enabled us to identify synthetic compounds that not only inhibited the quorum sensors in the majority of the cells but also led to the formation of flat, undifferentiated biofilms that eventually detached (60). It is notable that the synthetic furanones, in concentrations that significantly lower quorum sensing–controlled gene expression in planktonic cells, were equally active against biofilm bacteria, despite the profoundly different modes of growth. In contrast, classical antibiotics used to treat *P. aeruginosa* infections, e.g., tobramycin and piperacillin, are required at concentrations 100- to 1,000-fold higher in order to kill biofilm bacteria than in order to kill their planktonic counterparts. In addition, we observed that furanone-treated biofilms were more susceptible to killing by tobramycin than their untreated counterparts (61) (Figure 3).

In a recently published report, we used DNA array technology to demonstrate that furanone compounds specifically repress expression of quorum sensing–controlled genes in *P. aeruginosa* (61). Microarray analysis of wild-type *P. aeruginosa* PAO1 showed that expression of 93 genes (1.7% of the *P. aeruginosa* genome) was affected by the addition of the furanone compound C-30. Overall, 85 genes (1.5%) were repressed and eight genes (0.1%) were activated in response to C-30. Genes encoding multidrug efflux pumps and transporters were predominant among the induced genes. The furanone-repressed genes included many previously known as quorum sensing–regulated genes, including numerous *P. aeruginosa* virulence factor genes such as *lasB* (encoding elastase), *lasA* (encoding LasA protease), *rhlAB* operon (regulating rhamnolipid production), *phzA-G* operon (encoding phenazine biosynthesis), *hcnABC* operon (regulating hydrogen cyanide production), and *chiC* (encoding chitinase). To determine whether the remaining furanone-repressed genes were in fact controlled by quorum sensing, parallel mapping of the quorum-sensing regulon was performed using a *lasI rhlI* double mutant grown with or without AHL signals. A comparative analysis showed that 80% of the furanone-repressed genes are indeed controlled by quorum sensing. Furthermore, furanone-repressed genes are not restricted to quorum sensing–regulated genes controlled by one of the two *P. aeruginosa* quorum-sensing circuits but may be controlled by either or both of the circuits (61). Microarray analysis demonstrated that expression of the

lasI/lasR and *rhlI/rhlR* gene clusters, which encode the central components of the *P. aeruginosa* quorum-sensing system, was not notably affected by furanone treatment. This observation suggests that the furanone does not interfere with some of the regulatory systems controlling transcription of the *lasRI* and *rhlRI* genes, but rather that the furanone acts on these quorum-sensing regulators at the post-transcriptional level. Several genes involved in the biosynthesis of the *Pseudomonas* quinolone signal (PQS), including the *phnAB* operon and *pqsH* (64), were also repressed by the furanone compound. *Pseudomonas* quinolone signaling has been demonstrated, in concert with the AHL-based quorum-sensing systems, to be involved in the regulation of virulence factor production, in particular phenazine, pyocyanin, and hydrogen cyanide, and in autolysis of *P. aeruginosa* colonies (65).

A recent study has pointed at PQS signaling as an important regulatory function involved in *P. aeruginosa* adaptation and persistence in the cystic fibrotic lung environment (66). A pulmonary mouse infection model was used to study the effect of furanone compounds on the persistence of *P. aeruginosa* in chronic infections. Groups of mice infected with *P. aeruginosa* received subcutaneous furanone injections for 3 days, and this treatment was found to significantly reduce the bacterial load compared with that of the control group (67). Furthermore, the efficiency of bacterial clearing was positively correlated to the concentration of the furanone compound. The concentration used (as calculated by the whole-body concentration) was equal to or less than the concentrations required to inhibit expression of virulence factors in planktonic cultures and promote sloughing of in vitro biofilms.

Discussion

The purpose of research in this field has been to provide evidence that there are alternatives to the traditional mode of fighting bacterial infection. It is possible, from nature's own rich collection of chemical compounds, to generate powerful antipathogenic drugs that do not, per se, inhibit growth but instead interfere directly with microbial activity. The key to this concept is bacterial cell-to-cell communication. Knowledge of the molecular mechanisms underlying these signaling systems and their control of virulence, biofilm formation, and pathogenicity brings a completely new perspective to the potential control of microbial activity. Current halogenated furanones are too reactive, and therefore presumably too toxic, for the treatment of bacterial infections in humans. However, their proven ability to control *P. aeruginosa* infections in animal models is of considerable importance, since it demonstrates that quorum sensing is a useful and promising drug target in vivo. On the other hand, several obvious disadvantages are associated with AHL-based quorum-sensing antagonists. First, each antagonist has a narrow spectrum, and, therefore, specific antagonists have to be developed for each organism targeted. This might, however, prove advantageous in some scenarios. For instance, it would theoretically be possible to attenuate a single, pathogenic organism living in a mixed population of normal bacterial flora by a specific inhibitor while leaving the rest of the bacterial population unaffected. Second, the therapeutic use of AHL-based antagonists is complicated by the fact that some AHL signal molecules function as virulence factors per se, as they possess immunomodulatory activities and affect muscle tissue as well as tracheal gland cells.

The ability to control *P. aeruginosa* with antipathogenic drugs holds great promise that a whole range of opportunistic, pathogenic bacteria can be controlled by similar pharmaceuticals. *P. aeruginosa* is an attractive model organism for such studies, partly because of the recent development of suitable cDNA microarray technology by Affymetrix Inc. (Santa Clara, California, USA). We have used this technique to demonstrate the target specificity of certain first-generation antipathogenic drugs (61). We envision that this approach will be used in many primary research and pharmaceutical laboratories in the future quest for drugs that target specific cellular components or interactions. Given the large number of bacteria that employ quorum-sensing communication systems, chemical attenuation of unwanted bacterial activities rather than bactericidal or bacteriostatic strategies may find application in many different fields, e.g., in medicine, agriculture, and food technology. This new concept is highly attractive because it is unlikely to pose a selective pressure for the development of resistance. The present approach is therefore generic in nature and highly promising for defense against bacterial biofilms encountered in many infectious diseases, on medical implants, and in many industrial facilities and water pipelines.

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EXHIBIT B

ACS chemical biology

Bacterial Evolution by Intelligent Design

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ABSTRACT In a process called quorum sensing, bacteria produce and secrete certain signaling compounds (called autoinducers) that bind to receptors on other bacteria and activate transcription of certain genes. A clever genetic selection yields a new quorum-sensing transcriptional regulator that marches to the beat of a different drummer.

In the past decade, interest in engineering new strains of bacteria that are optimized to carry out processes of medical, agricultural, or industrial importance has exploded. This body of research, sometimes referred to as "synthetic biology", draws on decades of fundamental studies about the molecular biology of bacteria and seeks to exploit this knowledge to fine-tune existing types of bacterial physiology or to create whole new types of physiology. An excellent example of this research was recently published in *Nature Biotechnology* that described the isolation of a mutation in the quorum-sensing regulator LuxR of *Vibrio fischeri*. The mutation blocks the detection of the native chemical signal of *V. fischeri* but allows detection of a new signal that is not detected by the wild-type protein (*1*). This study will have applications in bacterial engineering and will provide insight about bacterial genetics, the detection of environmental signals, structural biology, and protein evolution.

Many types of bacteria communicate *via* the release and detection of diffusible chemical signals. These chemicals, which can be thought of as bacterial pheromones, stimulate diverse behaviors, including bioluminescence; the horizontal transfer of DNA; the formation of biofilms; and the production of pathogenetic factors, antibiotics, and other secondary metabolites (*2*). Gram-positive bacteria typically communicate by using oligopeptide signals that are detected by two-component phosphorelay proteins (*3*), whereas proteobacteria generally signal *via* acyl-homoserine lactones (AHLs). Additional classes of bacterial pheromones have also been described (*4*). In addition to using species-specific signals, some groups of bacteria appear to employ a universal signal (a bacterial Esperanto) to communicate intergenerically (*5*).

V. fischeri is a bioluminescent marine bacterium that symbiotically colonizes various species of fish and invertebrates, which in turn exploit bacterial luminescence for a variety of purposes (*5*). A protein called LuxI synthesizes 3-oxo-hexanoyl-L-homoserine lactone (OHHL), an AHL-type signal molecule (*6*), and a protein called LuxR is the signal sensor and a signal-dependent transcriptional activator of the luciferase operon (*7*, *8*). As a population of *V. fischeri* cells grows, the concentration of OHHL increases as a function of cell-population density. When the concentration of OHHL reaches the micromolar range, its passive efflux from the cells becomes balanced by a passive influx; therefore, its intracellular concentration increases enough to bind to LuxR. LuxR–OHHL complexes bind luciferase promoters and activate their transcription (Figure 1, panel a).

Intercellular signaling is thought to allow an estimation of population densities, a phenomenon sometimes referred to as quorum sensing (*9*). Certain bacterial behaviors are appropriate only if carried out simultaneously by large numbers of bacterial cells (a quorum). Bacteria are thought to use the concentration of these chemical signals as an indication of population density. However, this view of signaling is somewhat facile and teleological. One author suggested that these molecules might be released by a single bacterium to detect and

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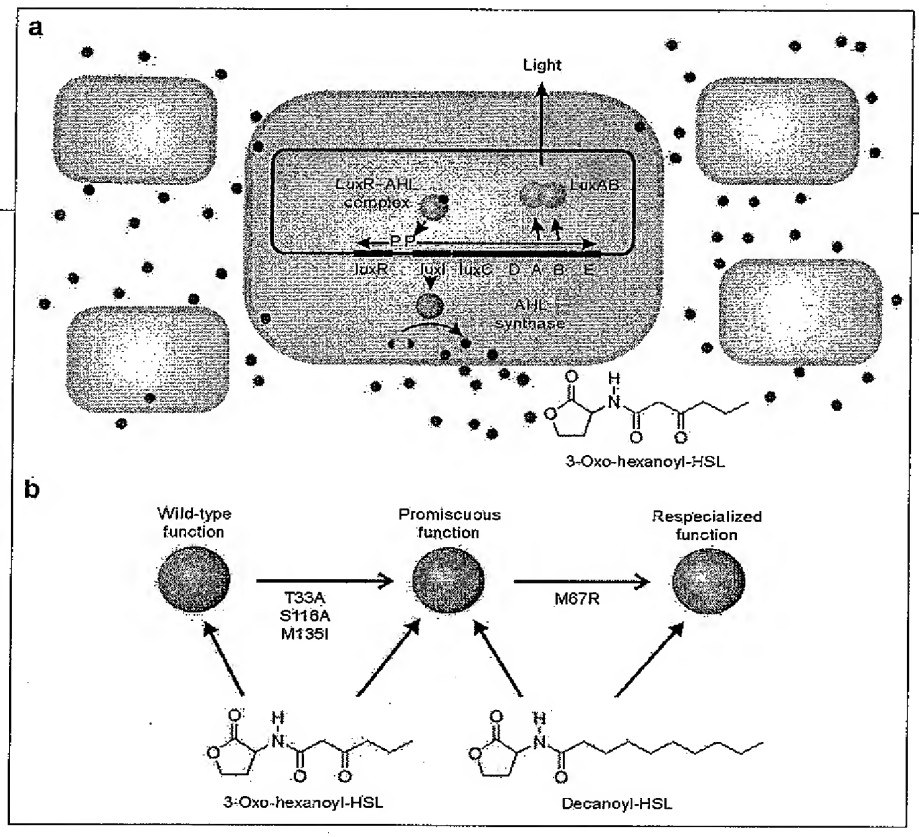

Figure 1. Communication in *V. fischeri*. a) Population-density-dependent expression of the luciferase operon of *V. fischeri*. LuxI proteins synthesize OHHL, which diffuses freely across the cell envelope. A combination of high cell density and a diffusion barrier causes these pheromones to accumulate and to bind to the LuxR transcription factor; this activates transcription of the luciferase operon, and bioluminescence results. b) Two-step evolution of a LuxR variant with altered AHL specificity. In the first step, a mutant was isolated that has a broadened specificity for AHLs; it detected both OHHL and decanoyl-HSL (as well as several other AHLs). In the second step, a mutant was isolated that detects the latter AHL but not the former.

measure diffusion barriers (*10*). According to this idea, these signal molecules can accumulate only if their diffusion is limited, and the bacterium might use this information, for example, to release hydrolytic enzymes or a matrix needed for biofilm formation. Both ideas are probably equally valid, because population density and diffusion barriers should both contribute to the accumulation of these signals. Equally plausible is that bacteria might require a quorum to effectively send and receive chemical signals; however, the goal of signaling may not be to measure the quorum but rather to coordinate the behavior of its members (*11*). In short, we should not pretend to understand the "why" of these bacterial signaling systems.

The study in *Nature Biotechnology* begins with a triple mutant in LuxR (referred to as G2E) that has the mutations T33A, S116A, and M135I; this shows a relaxed specificity of AHL signal molecules (*12*). Whereas the native LuxR detects primarily OHHL, the G2E protein can detect a wide variety of similar signals. In the present study, Collins and coworkers started with the G2E mutation and used a clever sequential positive and negative selection for mutants that can detect decanoyl-homoserine lactone (HSL) but not OHHL. They constructed a P*luxI–chloramphenicol acetyl transferase (cat)* fusion to select for function in the presence of decanoyl-HSL. Expression of this fusion causes chloramphenicol resistance and was used to select for LuxR function in the presence of decanoyl-HSL. Colonies were pooled, and their DNA was extracted and introduced into a strain containing a P*lux-bit* fusion, where *bit* inhibits the activity of β-lactamase. Expression of Bit in the presence of OHHL was counterselected on a medium that contained ampicillin. The surviving mutants all shared the same alteration, an arginine at position 67 in place of the wild-type methionine (M67R). It is unlikely that this mutation could readily have been isolated without this novel two-step selection. This LuxR variant can detect several AHLs with unsubstituted acyl groups but does not detect AHLs with 3-oxo substitutions.

Of the three alterations in mutant G2E, M135A is particularly interesting. Residue M135 lies at the same position as Ser126 of the TraR protein of *Agrobacterium tumefaciens*. The structure of TraR has been solved by X-ray crystallography (*13*). Ser126 of TraR lies close to Thr129, which makes a water-mediated hydrogen bond to the 3-oxo group of 3-oxo-octanoylhomoserine lactone (OOHL) (*13*). LuxR has a serine at position 137, which could play a similar role, and if so, the M135A mutant could destabilize this bond, thereby decreasing specificity for the 3-oxo group. Similarly, Met67 lies at the same position as Gln58 of the TraR. The β-carbon of Gln58 contacts the terminal carbon of the acyl chain of OOHL. This suggests that the β-carbon of Met67 of LuxR might hinder the binding of AHLs with acyl chains that are greater than six carbons, whereas the mutation M67R somehow relieves this block. This interpretation is consistent with the way the mutation was isolated (selection for detection of a long-chain unsubstituted AHL, and selection against detection of a short-chain 3-oxo-substituted AHL). How this mutation blocks the detection of the 3-oxo substitution is therefore puzzling. Perhaps future studies will solve this riddle.

As pointed out in the study, the isolation of the double mutant of LuxR may mimic the stepwise accumulation of mutations in nature. Several studies have shown that the first step in protein evolution often involves the acquisition of "promiscuous functions" that retain but broaden the ancestral functions (*14*). Later steps in evolution cause the protein to acquire "respecialized" proper-

ties. The original triple mutant caused the protein to detect a wide variety of AHLs, whereas the second mutation (M67R) restricted the detection of AHLs. Of course, the natural evolution of a new LuxR/LuxI-type regulatory system requires that both proteins coevolve such that the AHL synthase always makes a signal that the AHL receptor can detect. One AHL synthase and one AHL receptor were subjected to site-directed mutagenesis to create variants with new signal specificities. A T140A mutant of EsaI (a LuxI homologue) preferentially synthesizes an unsubstituted AHL rather than a 3-oxo substituted AHL (*15*). Similarly, a T129A or T129V mutant of TraR detected unsubstituted AHLs with the same affinity as 3-oxo AHLs (*16*). Curiously, overproduction of either protein also reduces its AHL specificity. Overproduction of AHL synthases is thought to deplete the cell of the favored acyl-carrier protein substrate, and the enzyme then uses less favored ones (*17*). Overproduction of several AHL receptors dramatically broadens their substrate specificities, though the reason for this is not clear (*18*).

The creation of a new signal specificity will have interesting and unforeseen applications in synthetic biology. In previous studies, geneticists have separated signal synthesis and detection in two different bacterial strains, so that one strain detects a signal sent by another. The current study will enable reciprocal signaling, such that one strain releases a signal that is detected by a second strain, which then synthesizes a second signal that is detected only by the first strain. Reciprocal signaling could be useful in constructing oscillating circuits through negative feedback or could control the timing of sequential steps in the biosynthesis of a complex pharmaceutical. Creating similar systems with three or more signals should be possible. In addition to the construction of new variants of LuxR, many natural LuxR homologues have been discovered that detect diverse AHLs and bind different DNA sequences. Researchers should be able to mix and match the binding domains of AHL and DNA of the various LuxR homologues to construct proteins that bind to new DNA sequences that are chosen by the intelligent bacterial designer.

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EXHIBIT C

Quorum Sensing: Cell-to-Cell Communication in Bacteria

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Key Words

autoinducer, quorum quenching, regulon

Abstract

Bacteria communicate with one another using chemical signal molecules. As in higher organisms, the information supplied by these molecules is critical for synchronizing the activities of large groups of cells. In bacteria, chemical communication involves producing, releasing, detecting, and responding to small hormone-like molecules termed autoinducers. This process, termed quorum sensing, allows bacteria to monitor the environment for other bacteria and to alter behavior on a population-wide scale in response to changes in the number and/or species present in a community. Most quorum-sensing-controlled processes are unproductive when undertaken by an individual bacterium acting alone but become beneficial when carried out simultaneously by a large number of cells. Thus, quorum sensing confuses the distinction between prokaryotes and eukaryotes because it enables bacteria to act as multicellular organisms. This review focuses on the architectures of bacterial chemical communication networks; how chemical information is integrated, processed, and transduced to control gene expression; how intra- and inter-species cell-cell communication is accomplished; and the intriguing possibility of prokaryote-eukaryote cross-communication.

Contents

QUORUM SENSING

Quorum-sensing bacteria produce and release chemical signal molecules termed autoinducers whose external concentration increases as a function of increasing cell-population density. Bacteria detect the accumulation of a minimal threshold stimulatory concentration of these autoinducers and alter gene expression, and therefore behavior, in response. Using these signal-response systems, bacteria synchronize particular behaviors on a population-wide scale and thus function as multicellular organisms. Here, we describe some well-characterized quorum-sensing systems with the aim of illustrating their similarities and differences. We presume similarities in these systems exist because the ability to communicate is fundamental to bacteria. Differences in the systems likely exist because each system has been optimized to promote survival in the specialized niche in which a particular species of bacteria resides. Thus, the types of signals, receptors, mechanisms of signal transduction, and target outputs of each quorum-sensing system reflect the unique biology carried out by a particular bacterial species.

Quorum Sensing in Gram-Negative Bacteria

The first described quorum-sensing system is that of the bioluminescent marine bacterium *Vibrio fischeri*, and it is considered the paradigm for quorum sensing in most gram-negative bacteria (Nealson & Hastings 1979). *V. fischeri* colonizes the light organ of the Hawaiian squid *Euprymna scolopes*. In this organ, the bacteria grow to high cell density and induce the expression of genes required for bioluminescence. The squid uses the light provided by the bacteria for counterillumination to mask its shadow and avoid predation (Visick et al. 2000). The bacteria benefit because the light organ is rich in nutrients and allows proliferation in numbers unachievable in seawater. Two

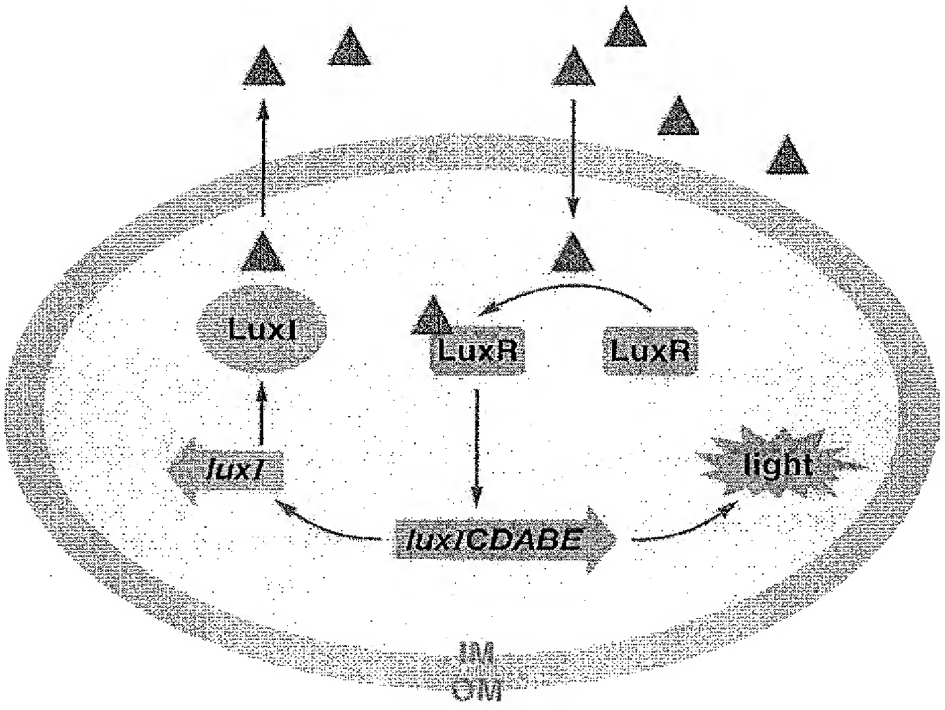

Figure 1
Quorum sensing in *Vibrio fischeri*; a LuxIR signaling circuit. Red triangles indicate the autoinducer that is produced by LuxI. OM, outer membrane; IM, inner membrane.

proteins, LuxI and LuxR, control expression of the luciferase operon (*luxICDABE*) required for light production (**Figure 1**). LuxI is the autoinducer synthase, which produces the acyl-homoserine lactone (AHL) autoinducer 3OC6-homoserine lactone (**Figure 2*a*** and Eberhard et al. 1981, Engebrecht & Silverman 1984), and LuxR is the cytoplasmic autoinducer receptor/DNA-binding transcriptional activator (Engebrecht et al. 1983). Following production, the AHL freely diffuses in and out of the cell and increases in concentration with increasing cell density (Kaplan & Greenberg 1985). When the signal reaches a critical, threshold concentration, it is bound by LuxR and this complex activates transcription of the operon encoding luciferase (Stevens et al. 1994). Importantly, the LuxR-AHL complex also induces expression of *luxI* because it is encoded in the luciferase operon. This regulatory configuration floods the environment with the signal. This creates a positive feedback loop that causes the entire population to switch into "quorum-sensing mode" and produce light.

A large number of other gram-negative proteobacteria possess LuxIR-type proteins and communicate with AHL signals (Manefield & Turner 2002). These systems are used predominantly for intraspecies communication as extreme specificity exists between the LuxR proteins and their cognate AHL signals. LuxI-type proteins link and lactonize the methionine moiety from *S*-adenosylmethionine (SAM) to particular fatty acyl chains carried on acyl-acyl carrier proteins (More et al. 1996, Parsek et al. 1999). A diverse set of fatty acyl side chains of varying length, backbone saturation, and side-chain substitutions are incorporated into AHL signals; these differences are crucial for signaling specificity (**Figure 2*a*** and Fuqua 1999). Structural studies of LuxI-type

Quorum sensing: a process of cell-cell communication in bacteria

Autoinducers: small molecules secreted by bacteria that are used to measure population density

AHL: acyl-homoserine lactone

SAM: *S*-adenosyl-methionine

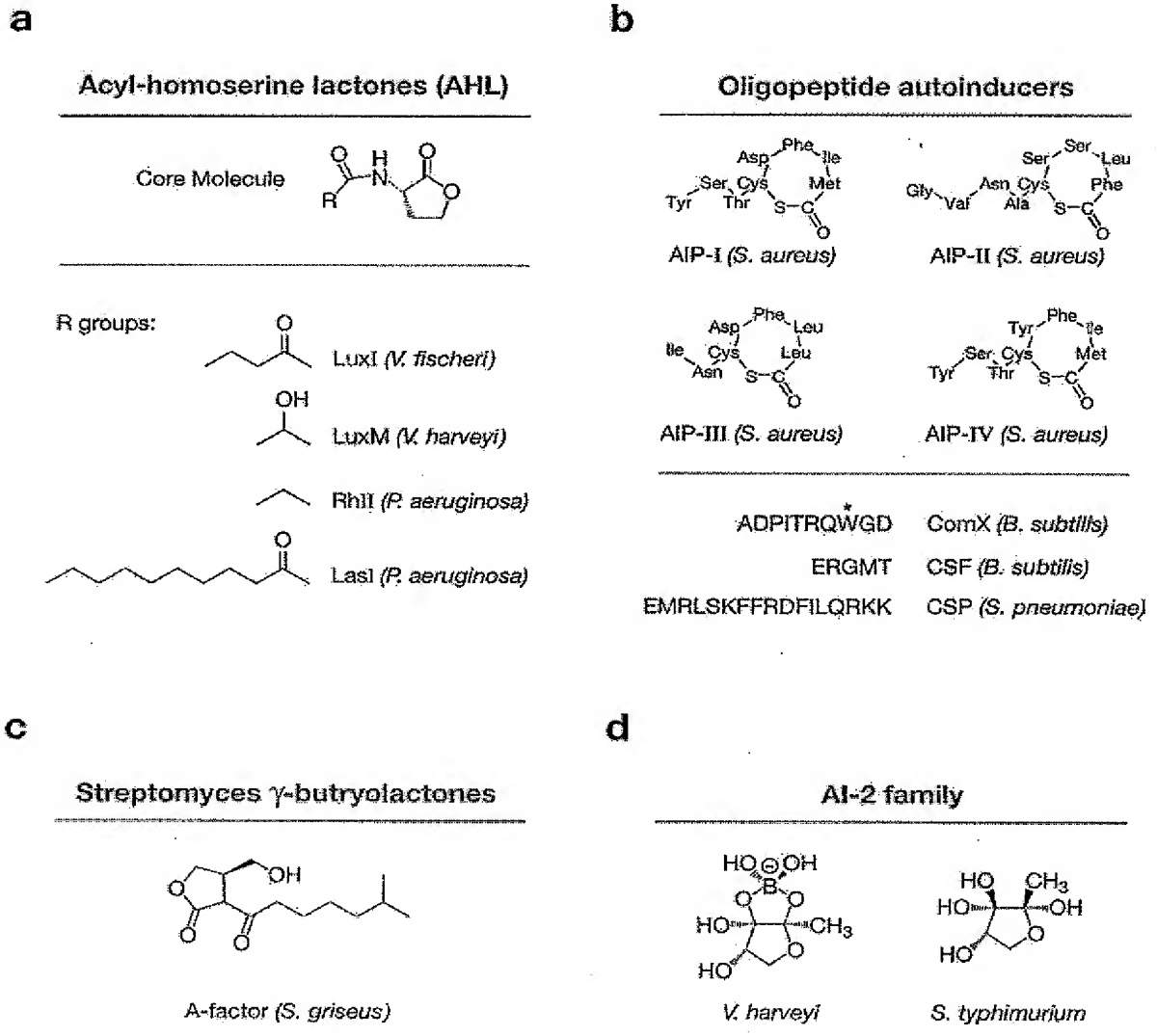

Figure 2

Representative bacterial autoinducers. The asterisk above the tryptophan in ComX represents an isoprenyl modification.

proteins indicate that each possesses an acyl-binding pocket that precisely fits a particular side-chain moiety (Gould et al. 2004, Watson et al. 2002). This structural feature apparently confers specificity in signal production. Thus, each LuxI protein produces the correct signal molecule with high fidelity. There are some LuxI-type proteins that produce multiple AHLs, although it is not clear if all are biologically relevant (Marketon et al. 2002). The structures of LuxR proteins suggest that LuxR proteins also possess specific acyl-binding pockets that allow each LuxR to bind and be activated only by its cognate signal (Vannini et al. 2002, Zhang et al. 2002b). Hence, it appears that in mixed-species

environments in which multiple AHL signals are present, each species can distinguish, measure, and respond only to the buildup of its own signal. Importantly, bacteria rarely rely exclusively on one LuxIR quorum-sensing system. Rather, bacteria use one or more LuxIR systems, often in conjunction with other types of quorum-sensing circuits.

Mechanisms must exist to prevent premature activation of LuxIR-type quorum-sensing circuits because both the signal and the detector are synthesized and interact in the cytoplasm (**Figure 1**). One such mechanism, evidenced by the LuxR homologue TraR in the plant pathogen *Agrobacterium tumefaciens*, is the stability of LuxR-type proteins increases upon AI binding. In the absence of autoinducer, TraR has a half-life of a few minutes. However, in the presence of AHL, the half-life of TraR increases to over 30 minutes (Zhu & Winans 1999). The crystal structure of TraR predicts that AHL binding is required for folding of the nascent polypeptide (Zhang et al. 2002b), and indeed radiolabeled TraR was stabilized only when its cognate AHL was added prior to labeling of the protein (Zhu & Winans 2001). Hence, only when AHL accumulates to a significant concentration (both outside and inside the cell) can TraR bind it, fold, and initiate the quorum-sensing cascade. Another mechanism that prevents "short-circuiting" of LuxIR systems is active export of AHL signals (Pearson et al. 1999). When a significant concentration of signal has accumulated, which is indicative of high cell density, diffusion into the cell overwhelms export and thus engages the circuit. AHLs with long acyl side chains are thought to require active export to transverse the bacterial membrane (Pearson et al. 1999).

Quorum Sensing in Gram-Positive Bacteria

Gram-positive bacteria communicate using modified oligopeptides as signals and "two-component"-type membrane-bound sensor histidine kinases as receptors. Signaling is mediated by a phosphorylation cascade that influences the activity of a DNA-binding transcriptional regulatory protein termed a response regulator. Similar to the mechanisms by which gram-negative bacteria use LuxIR quorum-sensing systems, each gram-positive bacterium uses a signal different from that used by other bacteria and the cognate receptors are exquisitely sensitive to the signals' structures. Thus, as in LuxIR systems, peptide quorum-sensing circuits are understood to confer intraspecies communication. Peptide signals are not diffusible across the membrane, hence signal release is mediated by dedicated oligopeptide exporters. In most cases, concomitant with signal release is signal processing and modification. While the biochemistry underlying these events is poorly defined, it is known that most peptide quorum-sensing signals are cleaved from larger precursor peptides, which then are modified to contain lactone and thiolactone rings, lanthionines, and isoprenyl groups (Ansaldi et al. 2002, Booth et al. 1996, Mayville et al. 1999, Nakayama et al. 2001). Many gram-positive bacteria communicate with multiple peptides in combination with other types of quorum-sensing signals.

A fascinating example of peptide quorum sensing exists in *Staphylococcus aureus*, which is normally a benign human commensal but becomes a deadly pathogen upon penetration into host tissues (reviewed in Tenover & Gaynes 2000). *S. aureus* uses a biphasic strategy to cause disease: At low cell density, the bacteria express protein factors that promote attachment and colonization, whereas at high cell density, the bacteria repress these traits and initiate secretion of toxins and proteases that are presumably required for dissemination (reviewed in Lyon & Novick 2004). This switch in gene expression programs is regulated by the Agr quorum-sensing system (**Figure 3**). The system consists of an autoinducing peptide of *Staphylococcus aureus* (AIP) (**Figure 2*b***) encoded by *agrD* (Ji et al. 1995) and a two-component sensor kinase-response regulator pair, AgrC and AgrA, respectively

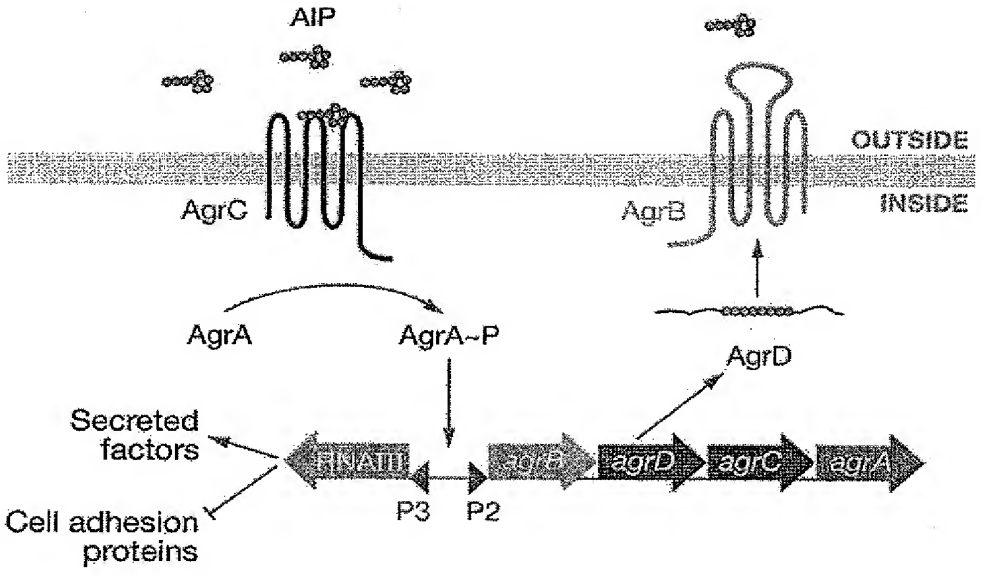

Figure 3

Using a two-component response regulatory system, *Staphylococcus aureus* detects and responds to an extracellular peptide. Small red circles indicate the AIP. P2 and P3 designate the promoters for *agrBDCA* and RNAIII, respectively.

AIP: autoinducing peptide of *Staphylococcus aureus*

(Novick et al. 1995). The AgrB protein exports and adds the thiolactone ring modification to *S. aureus* AIPs (Saenz et al. 2000). Binding of the AIP to AgrC leads to phosphorylation of AgrA. Phospho-AgrA induces the expression of a regulatory RNA termed RNAIII, which represses expression of cell-adhesion factors while inducing expression of secreted factors (Novick et al. 1993). Activated AgrA also induces expression of the *agrBDCA*. This results in increased AIP levels, which ensures that the entire population switches from the low-cell-density to the high-cell-density state (Novick et al. 1995).

S. aureus strains are classified on the basis of the sequence of their thiolactone-containing AIP. At present, four different AIPs (**Figure 2*b***) and Dufour et al. 2002), and thus four different groups of *S. aureus*, are known. Surprisingly, each AIP specifically activates its cognate AgrC receptor but inhibits activation of all others by competitive binding to the non-cognate receptors (Lyon et al. 2002b). Thus, each AIP inhibits activation of the virulence cascade in the other three groups of *S. aureus* while not affecting the other groups' growth. Coinfection with two different *S. aureus* groups results in intraspecies competition; the *S. aureus* group that first establishes its quorum-sensing cascade outcompetes the other group. Consistent with this idea, purified AIP II attenuates virulence of a Group I *S. aureus* in a mouse infection model (Mayville et al. 1999). Thus, in *S. aureus*, quorum sensing allows dissemination of closely related progeny while inhibiting the spread of non-kin. Clinical analyses show that each *S. aureus* group is the primary causative agent of a specific type of *S. aureus* disease. This suggests that cell-cell communication has been instrumental in establishing a specific niche for each "strain" (Novick 2003). The codivergence of the signal-receptor pairs occurring in these bacteria may be one molecular mechanism underlying the evolution of new bacterial species.

Streptomycetes are a diverse family of gram-positive soil-dwelling bacteria that are of clinical relevance because they are a major biological reservoir of secondary metabolites, many of which are used as antibiotics (reviewed in Chater & Horinouchi 2003). Streptomycetes use γ-butyrolactones (**Figure 2*c***) as autoinducers and control

morphological differentiation and secondary metabolite production via quorum sensing. Their signals are intriguing because they are structurally related to AHL autoinducers. However, there has not yet been any report describing either cross-communication between or cross-inhibition of streptomycetes and Gram-negative bacteria that communicate with AHLs.

QUORUM-SENSING NETWORK ARCHITECTURE

Identification of the chemical signals, receptors, target genes, and mechanisms of signal transduction involved in quorum sensing is leading to a comprehensive understanding of cell-cell communication in bacteria. This research is providing insight into the variety of molecular arrangements that enable communication between cells as well as the unique characteristics that the various signaling architectures provide in terms of information dissemination, detection, relay, and response. Below we highlight a few quorum-sensing systems and discuss how each particular network arrangement leads to distinct signaling features.

Parallel Quorum-Sensing Circuits

The first observation that bacteria could communicate with multiple quorum-sensing signals was in the quorum-sensing system of the Gram-negative, bioluminescent marine bacterium *Vibrio harveyi* (**Figure 4**). The *V. harveyi* quorum-sensing system consists of three autoinducers and three cognate receptors functioning in parallel to channel information into a shared regulatory pathway. Similar to other Gram-negative bacteria, *V. harveyi* produces an AHL signal termed HAI-1 (3OHC4-homoserine lactone; **Figure 2*a***and Cao & Meighen 1989). Its synthase, LuxM, shares no homology to LuxI-type enzymes but catalyzes the identical biochemical reaction to generate a specific AHL (Bassler et al. 1993, Hanzelka et al. 1999). HAI-1 binds to a membrane-bound sensor histidine kinase (LuxN) similar to sensors in Gram-positive quorum-sensing signaling circuits (Bassler et al. 1993, Freeman et al. 2000). The second *V. harveyi* signal is a furanosyl borate diester known as AI-2 (**Figure 2*d***and Bassler et al. 1994a, Chen et al. 2002), production of which requires the LuxS enzyme (Surette et al. 1999, Xavier & Bassler 2003). AI-2 is bound in the periplasm by the protein LuxP; the LuxP-AI-2 complex interacts with another membrane-bound sensor histidine kinase, LuxQ (Bassler et al. 1994a). The third *V. harveyi* signal, an unidentified molecule termed CAI-1, is produced by the CqsA enzyme, and again, this signal interacts with a membrane-bound sensor histidine kinase, CqsS (Henke & Bassler 2004b).

At low cell density, in the absence of appreciable amounts of autoinducers, the three sensors—LuxN, LuxQ, and CqsA—act as

CHEMICAL COMPLEXITY IN BACTERIAL AUTOINDUCERS

Recent research shows that a rich diversity of chemical molecules is used for communication in the bacterial world. New genetic, biochemical, and imaging techniques have enhanced our ability to identify and measure the readouts of cell-cell communication. These tools have led to the identification of several novel molecules and classes of molecules that are clearly bona fide autoinducers mediating cell-cell communication. A few examples are:

PQS. The molecule 3,4-dihydroxy-2-heptylquinoline, termed PQS, is a signal that is integral to the *P. aeruginosa* quorum-sensing cascade (Pesci et al. 1999). This signal acts as an additional regulatory link between the Las and Rhl quorum-sensing circuits.

3OH PAME. 3OH palmitic acid methyl ester (3OH PAME) transmits information via the two-component sensor histidine kinase-response regulator pair, PhcS-PhcR, to cause the plant pathogen *Ralstonia solanacearum* to switch from a motile to an infective state (Flavier et al. 1997).

CYCLIC DIPEPTIDES. Newly described in a number of gram-negative bacteria, at high concentrations, cyclic-dipeptides antagonize AHL binding to cognate receptors (Holden et al. 1999).

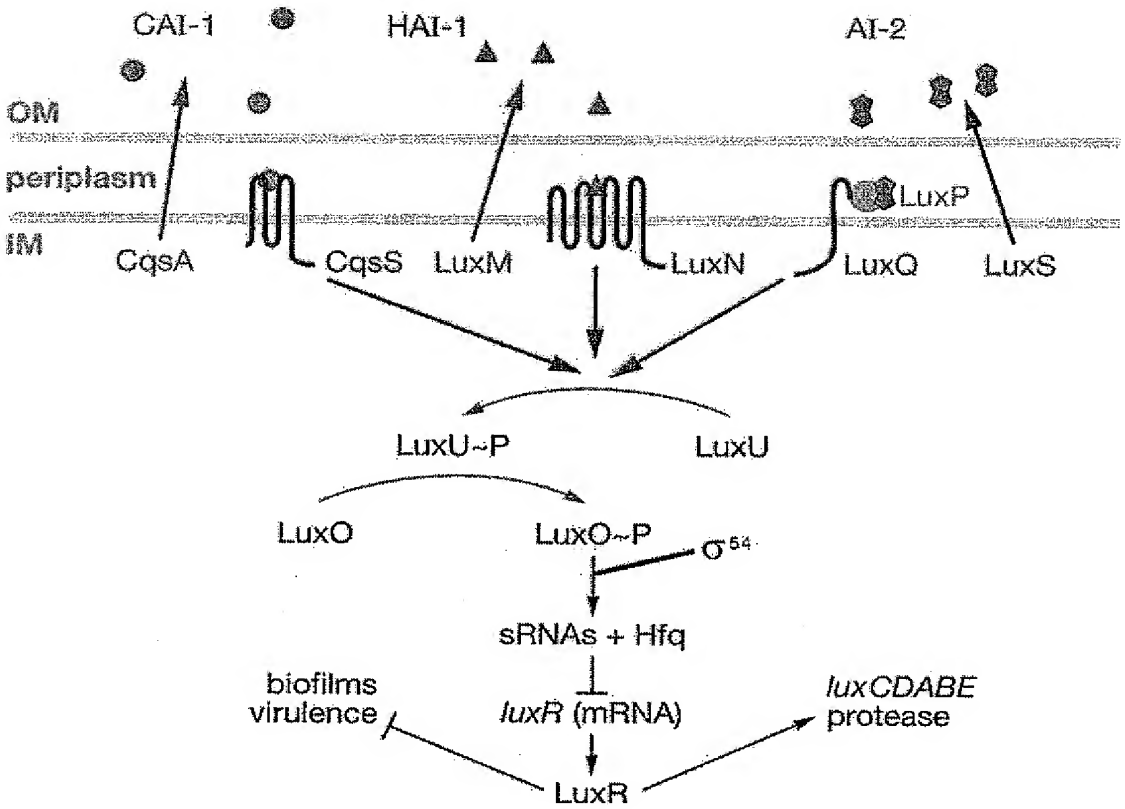

Figure 4

Vibrio harveyi produces and responds to three distinct autoinducers. The sensory information is fed into a shared two-component response regulatory pathway. The arrows indicate the direction of phosphate flow in the low-cell-density state. CAI-1, HAI-1, and AI-2 are respectively represented by green circles, red triangles, and blue double pentagons. OM, outer membrane; IM, inner membrane.

sRNAs: small RNAs

kinases, autophosphorylate, and subsequently transfer the phosphate to the cytoplasmic protein LuxU (Figure 4). LuxU passes the phosphate to the DNA-binding response regulator protein LuxO (Bassler et al. 1994b, Freeman & Bassler 1999a,b, Freeman et al. 2000). Phospho-LuxO, in conjunction with a transcription factor termed σ^{54}, activates transcription of the genes encoding five regulatory small RNAs (sRNAs) termed Qrr1–5 (for Quorum Regulatory RNA) (Lilley & Bassler 2000, Lenz et al. 2004). The Qrr sRNAs interact with an RNA chaperone termed Hfq, which is a member of the Sm family of eukaryotic RNA chaperones involved in mRNA splicing (Carrington & Ambros 2003). The sRNAs, together with Hfq, bind to and destabilize the mRNA encoding the transcriptional activator termed LuxR (not similar to LuxR of *V. fischeri*) (Lenz et al. 2004). LuxR is required to activate transcription of the luciferase operon *luxCDABE* (Swartzman et al. 1992). Thus, at low cell density, because the *luxR* mRNA is degraded, the bacteria do not express bioluminescence. At high cell density, when the autoinducers accumulate to the level required for detection, the three sensors switch from being kinases to being phosphatases and drain phosphate from LuxO via LuxU. Unphosphorylated LuxO

cannot induce expression of the sRNAs. This allows translation of *luxR* mRNA, production of LuxR, and expression of bioluminescence. This pathway controls many genes in addition to those encoding luciferase (Henke & Bassler 2004a, Mok et al. 2003).

The human pathogen *Vibrio cholerae*, the causative agent of the endemic diarrheal disease cholera, possesses a quorum-sensing network similar to that of *V. harveyi* (Miller et al. 2002). *V. cholerae* has no equivalent to the AI-1/LuxN branch of the system. However, this bacterium does possess the AI-2/LuxPQ and CAI-1/CqsS branches as well as LuxU, LuxO, four Qrr sRNAs, and a *V. harveyi* LuxR-like protein termed HapR. The *V. cholerae* systems function analogously to those of *V. harveyi* but control virulence instead of regulating bioluminescence (Miller et al. 2002, Zhu et al. 2002). Surprisingly, quorum sensing promotes *V. cholerae* virulence factor expression and biofilm formation at low cell density and represses these traits at high cell density (Hammer & Bassler 2003). Quorum sensing commonly controls bacterial virulence factor expression, but typically, induction occurs at high cell density. This opposite regulatory pattern exhibited by *V. cholerae* can be understood in terms of the specific disease that the bacterium causes. Following a successful *V. cholerae* infection, the ensuing diarrhea wash huge numbers of bacteria from the human intestine into the environment. Repression of virulence factor production and biofilm formation genes at high cell density may promote dissemination of *V. cholerae*.

Upon the recent completion of the *V. fischeri* genome sequence, it was revealed that, in addition to LuxIR, homologues of two of the *V. harveyi* quorum-sensing circuits and the shared downstream components are present: LuxMN, LuxSPQ, LuxU, LuxO, and LuxR (referred to as LitR in *V. fischeri*) (Fidopiastis et al. 2002, Lupp & Ruby 2004, Lupp et al. 2003, Miyamoto et al. 2003). In *V. fischeri*, the *V. harveyi*-like quorum-sensing systems activate expression of *litR* at low cell densities. LitR induces expression of *luxR*, which in turn promotes light production, as described above (Fidopiastis et al. 2002). This latter event occurs at relatively high cell densities, which presumably can be achieved only in the squid and cannot be achieved in the open ocean.

These three vibrio quorum-sensing systems underscore the way in which a common quorum-sensing network can be modified to fit the unique biology of the bacteria. Whereas the two-tiered *V. fischeri* circuit is adapted for two disparate lifestyles, inside and outside of the squid, *V. harveyi* and *V. cholerae* do not possess LuxIR homologues and they are not known to exist in symbiotic relationships. Although *V. harveyi* and *V. cholerae* share many of the same signals and receptors, the relative input from each signal is different in the two species. CqsA/CqsS is the dominant signaling-circuit in *V. cholerae* whereas it is the weakest in *V. harveyi* (Henke & Bassler 2004b). These signaling variations, coupled with their regulation of distinct downstream virulence factors, may be determining factors that allow *V. cholerae*, but not *V. harveyi*, to infect humans.

In each vibrio circuit, all signal-receptor pairs channel phosphate to LuxO in the absence of a signal and remove phosphate from LuxO in the presence of a signal. Thus, because all signals lead to a reduction in the level of LuxO-phosphate, each signal reinforces the information encoded in the other signals. This arrangement may allow the network to function as a coincidence detector that significantly activates or represses gene expression only when all signals are simultaneously present or absent (Mok et al. 2003). This signaling architecture may be critical for filtering out noise from molecules in the environment that are related to the true signals and/or noise from signal mimics produced by other bacteria in the vicinity.

Quorum-Sensing Circuits Arranged in Series

As in the vibrios, the *Pseudomonas aeruginosa* quorum-sensing circuit is responsive to

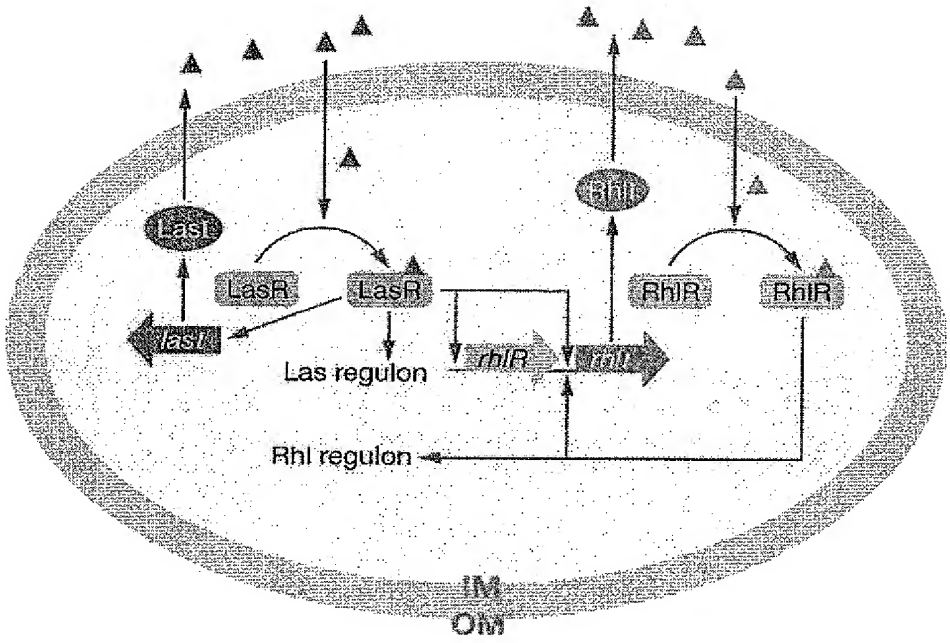

Figure 5

The *Pseudomonas aeruginosa* quorum-sensing circuits operate in series to control a large set of target genes. The LasI autoinducer is represented by red triangles and the RhlI autoinducer is shown as blue triangles. OM, outer membrane; IM, inner membrane.

CF: cystic fibrosis

multiple autoinducers, however, unlike those in vibrios, the *P. aeruginosa* regulatory systems are arranged in series rather than in parallel. *P. aeruginosa*, a common soil organism, is also an opportunistic pathogen most notorious for its devastating effects on cystic fibrosis (CF) patients (Eberl & Tummler 2004). Quorum sensing is essential for chronic *P. aeruginosa* respiratory infection because it controls adhesion, biofilm formation, and virulence factor expression, all of which allow persistence in the lung and are required for disease progression (Smith & Iglewski 2003).

The *P. aeruginosa* quorum-sensing network consists of two LuxIR circuits, termed LasIR and RhlIR (**Figure 5** and Gambello & Iglewski 1991, Ochsner et al. 1994). LasI, a LuxI homologue, produces an AHL autoinducer (3OC12-homoserine lactone; **Figure 2*a*** and Pearson et al. 1994) that binds to LasR. The LasR-autoinducer complex activates a variety of target genes including *lasI*, which sets up the characteristic positive feedback loop that further activates the system (**Figure 5** and Seed et al. 1995). The LasR-autoinducer complex also activates the expression of *rhlR* and *rhlI* encoding another quorum-sensing circuit. RhlI produces the AHL C4-homoserine lactone (**Figure 2*a***) and Pearson et al. 1995). Following accumulation, RhlR binds the RhlI-directed signal; this complex activates its own set of target genes. Importantly, because the LasIR system induces both *rhlI* and *rhlR*, induction of the genes under RhlIR control occurs subsequent to induction of genes under LasIR control.

Microarray analyses of *P. aeruginosa* quorum-sensing-controlled gene expression revealed three classes of genes: genes that respond to only one autoinducer, genes that respond to either autoinducer, and genes that require both autoinducers simultaneously for activation (Hentzer et al. 2003; Schuster et al. 2003; Wagner et al. 2003, 2004). Furthermore, transcriptome analyses showed that these classes of genes are expressed at different

times over the growth cycle. This indicates that the tandem network architecture indeed produces a temporally ordered sequence of gene expression that may be critical for the ordering of early and late events in a successful infection (Schuster et al. 2003, Whiteley et al. 1999).

Competitive Quorum-Sensing Circuits

The above quorum-sensing networks rely on multiple signals acting synergistically. Other quorum-sensing networks are arranged such that the signals antagonize one another. For example, *Bacillus subtilis* has two autoinducing peptides functioning in a network arrangement that allows *B. subtilis* to commit to one of two mutually exclusive lifestyles: competence (the ability to take up exogenous DNA) and sporulation (**Figure 6**). ComX, a 10-amino acid peptide (**Figure 2*b***) and Magnuson et al. 1994, Solomon et al. 1996) that is processed and secreted by ComQ (Bacon Schneider et al. 2002), is detected by the membrane-bound histidine sensor kinase ComP. ComX binding stimulates ComP to autophosphorylate and transfer phosphate to the DNA-binding response regulator ComA (Solomon et al. 1995). Phosphorylated ComA regulates transcription of a variety of genes encoding factors required for competence development (Nakano & Zuber 1991). A second oligopeptide autoinducer, competence and sporulation

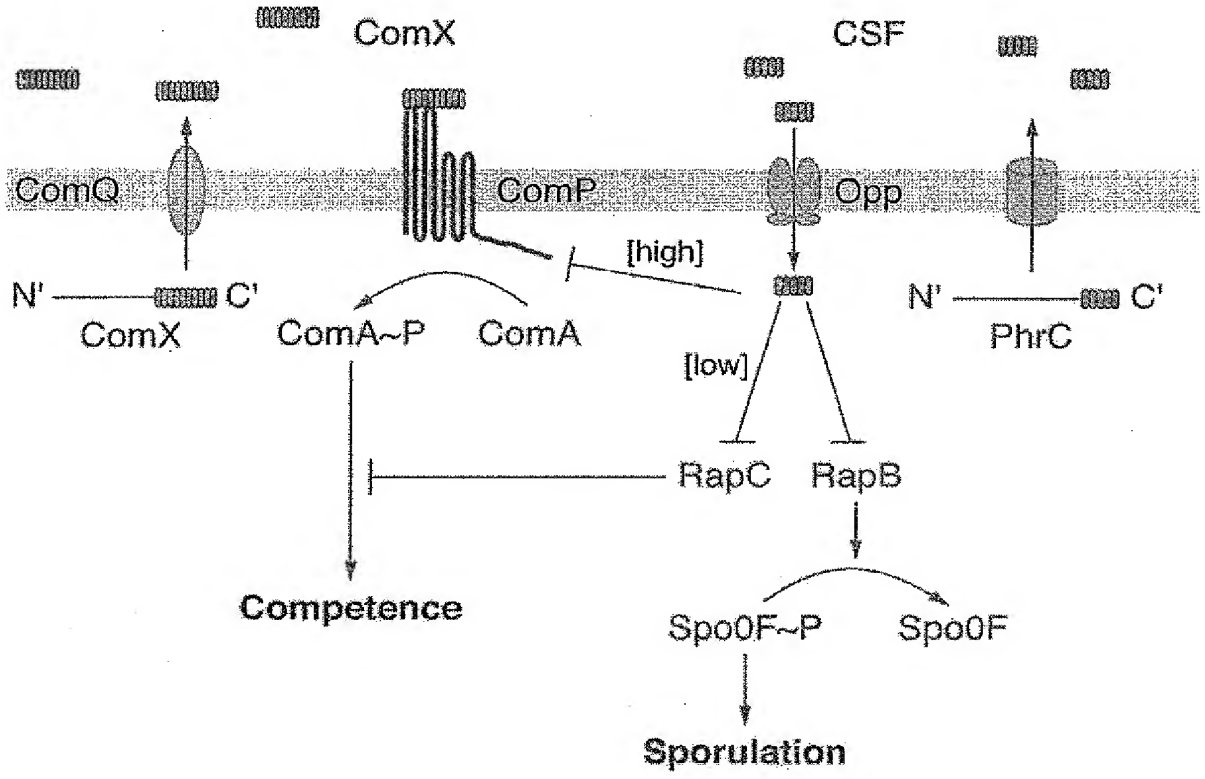

Figure 6

Bacillus subtilis produces two autoinducing peptides that regulate two different developmental pathways: competence and sporulation. ComX is represented as a chain of purple ovals and CSF is shown as a chain of red ovals.

CSF: competence and sporulation factor of *B. subtilis*

CSP: competence-stimulating peptide of *S. pneumoniae*

factor of *B. subtilis* (CSF; encoded by the gene *phrC*), is released via the general secretory apparatus, is re-internalized through the Opp peptide transporter, and acts in the cytoplasm (**Figure 2*b***; **Figure 6**; and Lazazzera et al. 1997, Solomon et al. 1996). At low internal concentrations, CSF binds to a protein named RapC and disrupts RapC binding to ComA (Perego 1997, Solomon et al. 1996). RapC binding to ComA inhibits competence development because DNA binding by ComA is prevented. Thus, CSF binding to RapC promotes competence development (Core & Perego 2003). However, at high concentrations, internalized CSF inhibits the ComP-ComA signaling cascade through an unknown mechanism, decreasing competence development and favoring sporulation (Lazazzera et al. 1997, Solomon et al. 1996). CSF also directly promotes sporulation by inhibiting RapB-mediated dephosphorylation of a response regulator named Spo0F, which, in its phosphorylated state, indirectly activates genes required for sporulation (Grossman 1995, Perego 1997).

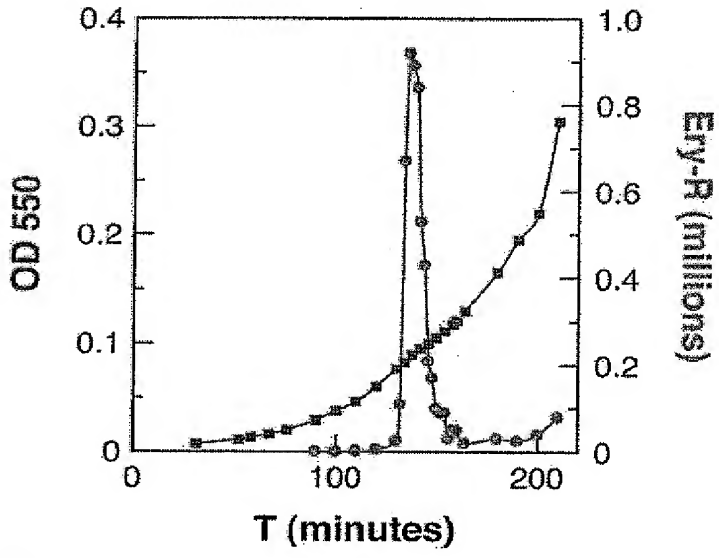

Figure 7

Competence in *Streptococcus pneumoniae* (*red line*) rises sharply at a specific growth stage (*blue line*), followed by a rapid decline. Optical Density (OD550) was used to measure cell number and resistance to erythromycin (Ery-R) was used to assess competence. Figure courtesy of D. Morrison.

Quorum-Sensing Circuits with On-Off Switches

The above quorum-sensing circuits allow bacteria to transition from a set of low cell density behaviors to a different set of high cell density behaviors. There are, however, quorum-sensing circuits that promote transient expression of particular traits followed by reversion to the original set of behaviors. Such an on-off switch controls competence development in the Gram-positive bacterium *Streptococcus pneumoniae*, which uses an oligopeptide autoinducer named competence-stimulating peptide (CSP) to monitor cell density (**Figure 2*b***). CSP is encoded by *comC* (Havarstein et al. 1995, Tomasz & Hotchkiss 1964). The transporter ComAB exports and modifies CSP (Hui et al. 1995). CSP is detected by the membrane-bound sensor histidine kinase ComD, which transfers phosphate to the cytoplasmic response regulator ComE (Pestova et al. 1996). This circuit controls the transcription of gene subsets in a precise temporal order. Early genes are expressed maximally 6–7 min after CSP accumulation; late genes are maximally induced at 9–10 min (Peterson et al. 2000). ComE directly activates transcription of early genes that include *comAB* and *comCDE*; this causes increased signal production and detection (Pestova et al. 1996). This positive feedback loop results in a dramatic, population-wide spike in competence when the bacteria reach the critical cell density (**Figure 7**). ComE also activates transcription of *comX*, a gene encoding an alternate sigma factor (Lee & Morrison 1999), and *comW*, which is required for transcription of late genes encoding proteins essential for DNA uptake (Luo et al. 2004).

A novel feature of *S. pneumoniae* quorum-sensing circuit is the rapidity with which the process of competence development initiates and terminates (**Figure 7** and Tomasz & Hotchkiss 1964). Importantly, competent

S. pneumoniae cells are more prone to autolysis than are noncompetent cells (Dagkessamanskaia et al. 2004, Morrison & Baker 1979, Seto & Tomasz 1975, Steinmoen et al. 2003). Thus, the benefit gained from acquiring DNA that can be used as a repository of new genes is maximized by efficiently activating and terminating the process to minimize lethality by autolysis. The events leading to termination have not been defined. It is known that ComX rapidly disappears when competence is terminated, which suggests that regulated proteolysis occurs (Luo et al. 2004).

Quorum-Sensing Systems Responsive to Host Cues

Agrobacterium tumefaciens induces crown gall tumors in plants through transfer and integration of a tumor-inducing (TI) plasmid into plant cells (Zhu et al. 2000). The tumors produce molecules termed opines, which the bacteria use as nutrients (Dessaux et al. 1992). The quorum-sensing circuit of *A. tumefaciens* is especially interesting because it is only activated at the host-bacterial interface owing to a requirement for both plant- and bacteria-produced signals. Mobilization of the TI plasmid is responsive to proximity to the plant because it requires detection of opines by a cytoplasmic receptor termed AccR or OccR (Beck von Bodman et al. 1992, Fuqua & Winans 1994). AccR/OccR-opine binding induces expression of the *V. fischeri*-like *luxR* homologue, *traR* (Fuqua & Winans 1994). TraR responds to an AHL autoinducer produced by the *V. fischeri* LuxI-type enzyme, TraI (Hwang et al. 1994, Zhang et al. 1993). Hence, bacterial number controls TI transfer because TraR bound to its autoinducer induces TI plasmid replication and bacterial-bacterial conjugation, which lead to increased infectivity of the population (Zhu & Winans 1999). Anti-TraR activators exist that limit TraR activity and thus presumably optimize the ratio of bacteria-bacteria to bacterial-plant TI transfer (Chai et al. 2001, Fuqua et al. 1995, Hwang et al. 1995).

GLOBAL CONTROL: QUORUM-SENSING REGULONS

TI: tumor-inducing

Regulon: a set of genes under common regulatory control

The advent of genomic profiling has shown that quorum sensing, in many bacteria, controls gene expression in a global manner. Two transcription profiling studies identified over 150 competence-regulated genes in *S. pneumoniae* that were categorized as early, late, delayed-induction, and repressed (Dagkessamanskaia et al. 2004, Peterson et al. 2004). As previously mentioned, early genes are required for signal production, export, and detection whereas some late genes are necessary for DNA internalization. Many of the delayed genes are involved in bacterial stress responses (Dagkessamanskaia et al. 2004, Peterson et al. 2004). Gene-disruption experiments analyzing 124 quorum-sensing-controlled genes found that only 23 are required for competence (Peterson et al. 2004). Quorum-sensing mutants of *S. pneumoniae* and related streptococci show defects in multiple pathways, including biofilm formation, acid tolerance, bacteriocin production, and virulence (reviewed in Suntharalingam & Cvitkovitch 2005). Together, these results suggest that quorum sensing in streptococcus controls the initiation of a global developmental program in which competence development represents only one aspect.

Further evidence that quorum sensing coordinates the control of a large subset of genes comes from transcriptome analyses of *P. aeruginosa* that identify 616 genes as part of the regulon. In one study, addition of autoinducers repressed 222 genes (Wagner et al. 2003). A concurrent study identified 315 quorum-sensing-controlled targets, of which only 38 were repressed (Schuster et al. 2003). Although the two experiments were performed under different growth and autoinducer conditions, the reasons for the discrepancies remain unclear. Importantly, prior to these profiling analyses, quorum-sensing-repressed targets had not been identified in *P. aeruginosa*. Similarly, transcriptional analysis of *V. cholerae* quorum-sensing mutants shows that

SAH: *S*-adenosylhomocysteine

DPD: 4,5-dihydroxy-2,3-pentonedione, a molecule generated by LuxS

the entire virulence regulon (>70 genes) is repressed by quorum sensing (Zhu et al. 2002).

These recent whole-genome quorum-sensing studies highlight two important ideas. First, quorum sensing allows bacteria to alternate between distinct genome-wide programs. These findings, along with an enhanced appreciation of the complexity of the quorum-sensing network architectures, have fundamentally changed the perception of bacteria as primitive single-celled organisms. Bacteria now are understood to undergo complex programs of development similar in many respects to eukaryotic organisms. Second, large groups of genes are repressed by quorum sensing. This finding challenges the notion that the primary function of quorum sensing is to initiate activities that are only beneficial to bacterial participation in group activities. Rather, an equally important function of quorum sensing may be to terminate processes that are only beneficial to bacteria living in relative isolation outside of a community structure.

INTERSPECIES COMMUNICATION AMONG BACTERIA

Beyond controlling gene expression on a global scale, quorum sensing allows bacteria to communicate within and between species. This notion arose with the discovery and study of the autoinducer AI-2, which is one of several signals used by *V. harveyi* in quorum sensing (**Figure 2*d*** and **Figure 4**). Specifically, *luxS* encoding the AI-2 synthase is present in roughly half of all sequenced bacterial genomes, AI-2 production has been verified in a large number of these species, and AI-2 controls gene expression in a variety of bacteria. Together, these findings have led to the hypothesis that bacteria use AI-2 to communicate between species (reviewed in Xavier & Bassler 2003).

LuxS functions in the pathway for metabolism of SAM, the major cellular methyl donor. Transfer of the methyl moiety to various substrates produces the toxic byproduct *S*-adenosylhomocysteine (SAH) (Schauder et al. 2001). In non-LuxS-containing bacteria and eukaryotes, the enzyme SAH hydrolase metabolizes SAH to adenosine and homocysteine. However, in bacteria containing LuxS, two enzymes, Pfs and LuxS, act sequentially to convert SAH to adenine, homocysteine, and the signaling molecule DPD (**Figure 8** and Schauder et al. 2001). DPD is a highly reactive product that can rearrange and undergo additional reactions, which suggests that distinct but related molecules derived from DPD may be the signals that different bacterial species recognize as AI-2. Two distinct DPD-derived signals were identified in *V. harveyi* and *Salmonella typhimurium* by trapping the active molecules in their respective receptors (LuxP for *V. harveyi* and LsrB for *S. typhimurium*), crystallizing the complexes, and solving their structures (Chen et al. 2002, Miller et al. 2004, Taga et al. 2001). In *V. harveyi*, AI-2 is (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF borate); in *S. typhimurium*, AI-2 is (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) (**Figure 2*d*** and **Figure 8**). Straightforward chemistry links these two molecules, as DPD can cyclize with two equally feasible stereochemistries. Following hydration and borate addition, the upper cyclization pathway in **Figure 8** yields the *V. harveyi* AI-2 and the lower cyclization and hydration pathway yields the *S. typhimurium* AI-2.

Identification of boron in *V. harveyi* AI-2 is surprising, as few biological roles for boron are known. However, boron is present in high concentrations (~0.4 mM) in the marine environment, which makes it a reasonable element in the *V. harveyi* AI-2 signal (Bowen 1966). Significantly lower boron concentration is found in terrestrial environments. This makes boron an unlikely component of the *S. typhimurium* AI-2 signal (Fresenius 1988). Importantly, all of the chemical species shown in **Figure 8** exist in equilibrium and rapidly

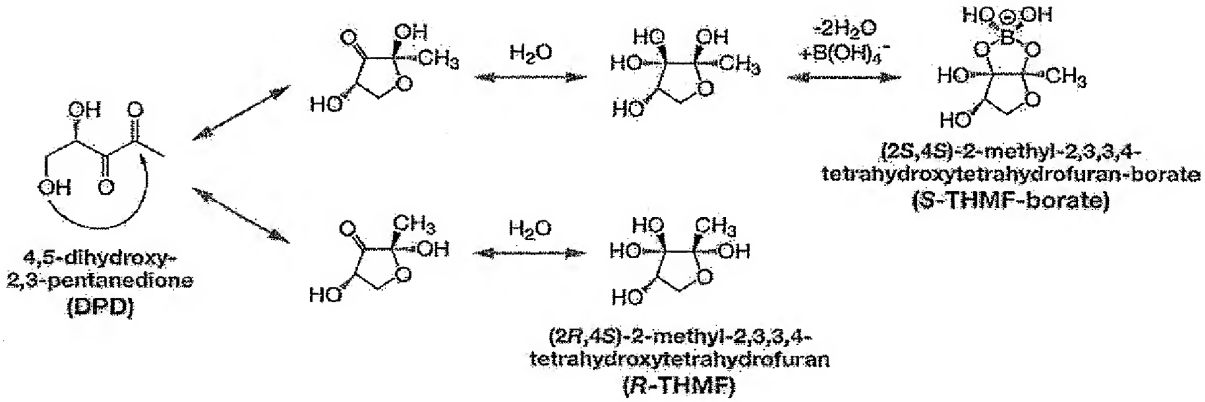

Figure 8

AI-2 is a family of interconverting molecules derived from DPD. *Vibrio harveyi* AI-2 is *S*-THMF-borate and *Salmonella typhimurium* AI-2 is *R*-THMF. Figure from Miller et al. 2004.

interconvert. Moreover, the concentrations of each molecule can be altered by manipulating the boron concentration. For example, addition of boron to DPD preparations promotes formation of the *V. harveyi* AI-2 molecule at the expense of the *S. typhimurium* signal. This shift is biologically relevant because DPD supplemented with boron causes *V. harveyi* to produce maximal bioluminescence whereas the same mixture inhibits the AI-2 response from *S. typhimurium*. Conversely, DPD preparations depleted for boron promote formation of the *S. typhimurium* signal with the concomitant loss of the *V. harveyi* AI-2. Again, this chemistry is borne out in the effect on AI-2-responsive gene expression in the two bacterial species (Miller et al. 2004).

These initial AI-2 investigations show that bacteria employ a conserved biosynthetic pathway to synthesize chemical signal intermediates whose fates are ultimately defined by the chemistry of the particular environment. Other DPD derivatives may exist and be biologically active. Additionally, some bacteria may possess two or more AI-2 receptors for recognition of different derivatives of DPD and alter particular behaviors in response to the information conveyed by each signal. Because only one enzyme (LuxS) is required to synthesize this family of interconverting signal molecules, this pathway may represent an especially economical method for evolving a complex bacterial lexicon.

QUORUM QUENCHING

The fundamental role of quorum sensing appears to be global control of the physiology of bacterial populations. This control is often exerted at the interface of different bacterial populations or at the bacterial-host margin. In niches in which bacterial populations compete for limited resources, the ability to disrupt quorum sensing may give one bacterial species an advantage over another that relies on quorum sensing. Likewise, a host's ability to interfere with bacterial cell-cell communication may be crucial in preventing colonization by pathogenic bacteria that use quorum sensing to coordinate virulence. Thus, it is not surprising that mechanisms have evolved to interfere with bacterial cell-cell communication in processes termed quorum quenching. Analogous mechanisms presumably exist for promoting quorum-sensing-controlled behaviors when such behaviors provide benefits to organisms cohabitating with quorum-sensing bacteria. These latter processes are not yet well defined, so we will focus our discussion primarily on mechanisms of quorum quenching.

Prokaryote-to-Prokaryote Quorum Quenching

As mentioned, cross-inhibition of AIP-mediated signaling in *S. aureus* represents a clear example of a quorum-quenching mechanism because each of the four AIPs specifically inhibits quorum sensing in competing *S. aureus* groups while not disrupting growth and other cellular functions (Lyon et al. 2002a). Many *Bacillus* species secrete an enzyme, AiiA, that cleaves the lactone rings from the acyl moieties of AHLs and renders the AHLs inactive in signal transduction (Dong et al. 2000). AiiA is extremely nonspecific with regard to the AHL acyl side chain, which suggests that this strategy interferes generically with AHL-mediated communication between gram-negative bacteria (Dong et al. 2001). Significantly, as previously mentioned, *Bacillus* relies on oligopeptide-mediated quorum sensing. Therefore, this tactic, while disrupting gram-negative bacterial communication, leaves *Bacillus* cell-cell communication unperturbed.

The soil bacterium *Variovorax paradoxus* uses a different generalized anti-AHL quorum-quenching tactic (Leadbetter & Greenberg 2000). Like *Bacillus*, *V. paradoxus* also degrades AHLs. However, in this case, AHL destruction occurs via an acylase-mediated lactone ring opening. *V. paradoxus* uses the linearized product of the reaction as a source of carbon and nitrogen. This strategy provides *V. paradoxus* with a double benefit: It terminates competitors' group behaviors and simultaneously increases its own growth potential (Leadbetter & Greenberg 2000). Particular *Ralstonia* isolates contain an AHL acylase encoded by *aiiD*, which suggests a similar anti-quorum-sensing mechanism to that of *V. paradoxus* (Lin et al. 2003). The *Ralstonia* quorum-sensing system is immune because the *Ralstonia* autoinducer, 3OH-PAME (Flavier et al. 1997), is not affected by AiiD activity (described in the sidebar).

In some cases, bacteria may degrade their own autoinducers, which presumably terminates quorum-sensing activities. For example, in stationary phase, *A. tumefaciens* produces the AttM AHL lactonase, which can degrade the *A. tumefaciens* autoinducer (Zhang et al. 2002a). It is hypothesized that it is disadvantageous for *A. tumefaciens* to continue to participate in group activities at this late growth stage and that AttM halts these processes. *Erwinia carotovora* and *Xanthomonas campestris* show a similar loss of AHL in stationary phase growth, which suggests an autoinducer degradative activity (Barber et al. 1997, Holden et al. 1998). *P. aeruginosa* degrades long, but not short, chain AHLs through an AiiD-type acylase named PvdQ (Huang et al. 2003). In this case, the RhlI autoinducer, C4-homoserine lactone, is immune, and the LasI autoinducer, 3OC12-homoserine lactone, can be destroyed. Interestingly, *pvdQ* is a member of the LasIR regulon and it is thus under 3OC12-homoserine control (Huang et al. 2003, Whiteley et al. 1999).

Some enteric bacteria, including *S. typhimurium* and *Escherichia coli*, import AI-2 with an AI-2-specific transporter (Surette et al. 1999; Taga et al 2001, 2003; Xavier & Bassler 2005). Once AI-2 is in the cytoplasm, a series of enzymatic reactions inactivates its signaling activity. This process reduces extracellular AI-2 concentrations to levels indicative of low cell density and—because AI-2 is used for interspecies communication—indicative of monospecies environments. AI-2 internalization is suspected to be another mechanism for interference with chemical communication among bacteria. Because *E. coli* and *S. typhimurium* also produce AI-2 and respond to this signal (Taga et al. 2001, 2003), it is not clear how these bacteria regulate AI-2 import while protecting the fidelity of their own AI-2 signaling cascades.

Eukaryote-to-Prokaryote Quorum Quenching

Several eukaryotic mechanisms that counteract bacterial quorum sensing have recently

been discovered. The Australian red macroagla *Delisea pulchra* coats its surface with a mixture of halogenated furanones that bear structural similarity to AHLs (Givskov et al. 1996). The furanones are internalized by bacteria, bind to LuxR-type proteins, and cause the degradation of these proteins (Manefield et al. 2002). This strategy prevents bacterial colonization of the algal surface by inhibiting quorum-sensing-controlled biofilm formation.

The legume *Medicago truncatula* controls over 150 proteins in response to AHLs produced by two model quorum-sensing bacteria; *Sinorhizobium meliloti* and *P. aeruginosa* (Mathesius et al. 2003). The plant secretes compounds in response to AHLs. These factors inhibit AI-2 signaling and stimulate AHL signaling in quorum-sensing reporter strains. The plant presumably encourages signaling between AHL-producing bacteria but not AI-2-producing bacteria because only the former are beneficial to the plant. Similarly, *Pisum sativum* (pea) produces AHL mimics that both positively and negatively affect AHL-regulated behaviors in a number of bacterial reporter strains (Teplitski et al. 2000).

Reactive oxygen and nitrogen intermediates generated by NADPH oxidase inactivate the *S. aureus* autoinducing peptide in a mouse air pouch skin model (Rothfork et al. 2004). These findings indicate a novel role for NADPH oxidase, an important component of innate immunity, in protection from bacterial infections. Consistent with this, mice deficient in NADPH oxidase have reduced resistance to infection by *S. aureus*, whereas infection from quorum-sensing mutant *S. aureus* remains unaffected by the loss of NAPH oxidase (Rothfork et al. 2004). This latter result suggests that reactive oxygen species influence infectivity only through quorum quenching. The authors of this study speculate that oxidation of other kinds of quorum-sensing molecules by NADPH oxidase is likely (Rothfork et al. 2004).

Human cells also have quorum-quenching activity. Analysis of primary and immortalized human epithelial cell lines show specific inactivation of the *P. aeruginosa* 3OC12-homoserine lactone autoinducer (the product of LasI) but not of the C4-homoserine lactone autoinducer (the product of RhlI) (Chun et al. 2004). Although presently uncharacterized, the quenching activity is membrane associated and heat liable, which suggests that it is a protein. This activity is intriguing in terms of the development of anti-*P. aeruginosa* therapies for treatment of CF.

Biotechnological Applications of Quorum Quenching

Naturally occurring quorum-quenching processes are being tested as novel antimicrobial therapies. Overexpression of *aiiA* in tobacco and potato plants confers resistance to *E. carotovora*, which requires AHL-controlled virulence factor expression to cause disease (Dong et al. 2001). Likewise, coculture of *Bacillus thuringiensis* decreased *E. carotovora*–mediated plant disease in an *aiiA*-dependent manner (Dong et al. 2004). Mice treated with synthetic antagonists of *S. aureus* AIP show resistance to infection (Mayville et al. 1999). Similarly, purified halogenated furanones appear to attenuate virulence of bacteria in mouse models (Hentzer et al. 2003, Wu et al. 2004). These and other examples predict that inhibition of quorum sensing offers an attractive alternative to traditional antibiotics because these strategies are not bactericidal and the occurrence of bacterial resistance therefore could be reduced. Likewise, approaches aimed at promoting beneficial quorum-sensing associations may enhance industrial-scale production of natural or engineered bacterial products.

EVOLUTION AND MAINTENANCE OF QUORUM SENSING IN BACTERIA

Quorum sensing presumably provides bacteria benefits from group activities that may be unattainable to an individual bacterium acting

alone. For example, the benefit derived from secretion of antibiotics or proteases may only occur when these exoproducts exceed a particular extracellular concentration, and achieving this concentration is only possible through the synchronous activity of a group of cells. The idea that bacteria cooperate has led to new questions regarding the evolution of cell-cell communication in bacteria, the cost bacteria pay for communicating, how fidelity is maintained in quorum-sensing systems, how cheating is controlled, and if and how "eavesdropping" occurs.

Although these evolutionary questions are new in the context of the molecular mechanisms underlying quorum-sensing-controlled behaviors, there exists an extensive literature dealing with these topics in other social organisms (Bourke 2001, Bradley 1999, Korb & Heinze 2004). For example, some social insects (e.g., ants and bees) have sterile worker castes that promote colony fitness even though the workers have no chance at reproduction (Queller & Strassmann 2002). The predominant explanation for these behaviors rests on Hamilton's kin-selection theory that predicts that even without directly contributing to reproduction, organisms belonging to a multimember group promote the inheritance of their own genes by increasing the fitness of closely related kin (Hamilton 1964a,b). A key component of kin selection is the ability to recognize another individual as kin. The ability to distinguish between and communicate with specific chemical signal molecules may enable a type of "kin selection" in bacteria. Consistent with this, many higher social organisms rely heavily on chemical signaling to maintain the integrity of the social order (Breed et al. 2004, Holldobler 1995, Queller & Strassmann 2002).

Cases of sacrifice of the individual for the group benefit also exist in microorganisms. For example, both the soil-dwelling bacterium *Myxococcus xanthus* and the slime mold *Dictyostelium discoidium*, in the absence of nutrients, produce resistant spores that survive nonvegetatively for long periods and can be dispersed to new environments (Dao et al. 2000, Strassmann et al. 2000). Spore development requires a large percentage of the population to undergo a lethal differentiation event that leads to structures whose function is to promote spore generation and dispersal. Chemical communication is required for these developmental events in both *D. discoidium* and *M. xanthus*: cAMP and Differentiation-Inducing Factor initiates development of fruiting bodies in *D. discoidium* (Konijn et al. 1969, Town & Stanford 1979, Town et al. 1976), whereas quorum-sensing communication controls the process in *M. xanthus* (Shimkets 1999).

Two examples exist to date that illustrate a selection for maintenance of quorum sensing. In *V. fischeri*, mutants incapable of luciferase production are outcompeted by the wild-type bacteria in the squid host. This indicates that the squid may possess a policing mechanism to eliminate cheater cells. Interestingly, the defect in these mutants was in luciferase itself, which suggests that the squid somehow distinguishes between cells that can and cannot contribute to light production (Visick et al. 2000). *A. tumefaciens*–induced plant tumors contain a large percentage of plasmid-free bacterial cells (Belanger et al. 1995). These cells have a faster growth rate and may therefore more efficiently grow on the opine nutrients produced in the plant tumors. However, plasmid-free bacteria are unable to initiate new tumor formation. Interestingly, as bacterial density increases and nutrients become limiting, increased bacterial-bacterial conjugation occurs and the TI plasmid is replicated to a higher copy number. Both of these events require quorum sensing and ensure that most of the bacteria acquire copies of the plasmid before they disseminate to a new location. This elegant strategy optimizes growth inside the tumor while maintaining the population's virulence and at least partially explains why quorum sensing is maintained.

Mechanisms for eavesdropping apparently also exist in quorum-sensing systems.

P. aeruginosa does not have *luxS* and therefore does not produce AI-2. However, *P. aeruginosa* detects AI-2 produced by the indigenous nonpathogenic microflora present in CF sputum samples (Duan et al. 2003). In the CF lung, *P. aeruginosa* exists in a complex microbial community composed of a variety of pathogenic and nonpathogenic bacteria. The detection of AI-2 may alert *P. aeruginosa* that *P. aeruginosa* is in the lung and that a program of gene expression that enhances persistence/virulence in the host is required. Consistent with this idea, CF sputum contains high concentrations of AI-2 and AI-2 induces *P. aeruginosa* virulence factor expression (Duan et al. 2003). In another example, *Salmonella enterica*, which has a *V. fischeri*–type LuxR-type protein (SdiA) but no LuxI-type enzyme, intercepts AHLs produced by other LuxI-containing gram-negative bacteria. In response to these signals, *S. enterica* expresses the *rck* operon and other genes that protect *S. enterica* from host defenses in the intestine (Ahmer et al. 1998). This result is interpreted to mean that the AHL signals signify that *S. enterica* is in a dense population of bacteria, which can presumably be attained only inside a host.

The ecological and evolutionary implications of quorum sensing in bacteria are only beginning to be addressed (Travisano & Velicer 2004). However, continued study of such questions hopefully will provide insight into the evolution and maintenance of group dynamics and behavior.

RHOMBOID: A SHARED PROKARYOTIC AND EUKARYOTIC CHEMICAL COMMUNICATION MECHANISM

New data suggest that some bacterial and eukaryotic signaling mechanisms have a common evolutionary origin. The inner membrane protein AarA of *Providencia stuartii* is required for the release of an extracellular quorum-sensing signal whose structure has not been defined (Rather et al. 1999). AarA has homology to the *Drosophila melanogaster* RHO (Gallio et al. 2002), which is a serine protease required for intramembrane cleavage, release, and activation of Epidermal Growth Factor receptor ligands (Klambt 2000, 2002). RHO is essential for many developmental processes in *D. melanogaster*, including proper wing vein development and organization of the fly eye (Schweitzer & Shilo 1997). Consistent with the idea that AarA and RHO have a common signaling function, expression of *P. stuartii aarA* in a *D. melanogaster rho* mutant rescued wing vein development. Likewise, expression of *rho* in a *P. stuartii aarA* mutant complemented the quorum-sensing signaling defect (Gallio et al. 2002). Homologues of RHO/AarA are nearly ubiquitous in all three kingdoms of life: bacteria, archea, and eukaryotes (Koonin et al. 2003). Five of eight tested bacterial Aar/RHO orthologues specifically cleaved RHO substrates, which suggests a widespread conservation of the mechanism of RHO with its bacterial homologues (Urban et al. 2002). These fascinating results show that bacteria and higher eukaryotes share a common cell-cell communication system; however, it has not been determined if any cross-kingdom communication can be mediated by RHO or its homologues.

RHO: rhomboid protein

A recent bioinformatics study suggests that the RHO/AarA finding is not an anomaly but rather that many signaling mechanisms may be shared by prokaryotes and eukaryotes. Enzymes involved in the production of cell-cell signaling molecules in vertebrates have homologues in bacteria but are absent from plants and archea (Iyer et al. 2004). A few of numerous examples are the enzymes phenylethanolamine N-methyltransferase (which catalyzes the conversion of norepinephrine to epinephrine), histidine decarboxylase (which catalyzes histidine to histamine), and glutamate decarboxylase (which catalyzes glutamate to γ-aminobutyric acid). It is hypothesized that eukaryotes acquired these genes from bacteria through a series of horizontal gene transfer

events (Iyer et al. 2004). These findings suggest that bacteria and eukaryotes share enzymes responsible for many cell-cell signaling pathways. This points to the exciting possibility that prokaryotic-to-eukaryotic cross-kingdom communication may be more prevalent than is currently appreciated.

CONCLUSIONS

It is now clear that cell-cell communication is the norm in the bacterial world and that understanding this process is fundamental to all of microbiology, including industrial and clinical microbiology. Our knowledge of quorum sensing may ultimately affect our understanding of higher-organism development. Quorum sensing was, until recently, considered to promote exclusively intraspecies communication and thus enable clonal populations of bacteria to count their cell numbers and alter gene expression in unison. While some autoinducers indeed appear to be extremely species-specific, new research shows that others are either genus-specific or promote intergenera communication. Further, hints that interkingdom communication occurs are becoming increasingly prevalent. Coincident with these findings are the beginnings of an understanding that prokaryotic and eukaryotic mechanisms that enhance and interfere with bacterial chemical communication also exist in nature. Bacterial quorum-sensing signal detection and relay apparatuses are complex and often consist of multiple circuits organized in a variety of configurations. Because bacteria routinely exist in fluctuating environments containing complex mixtures of chemicals, some of which are signals and some of which presumably do not convey meaningful information, we hypothesize that each quorum-sensing network organization evolved to solve the particular set of communication needs a particular species of bacteria encounters. Elements of these elegant solutions for deciphering complex chemical vocabularies appear to be conserved and used for analogous purposes in eukaryotes.

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This study supports the hypothesis that autoinducer binding is required for protein stability of LuxR-like AHL receptors and suggests a mechanism for how bacteria avoid premature activation of their quorum-sensing cascades.

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